ANTIGENOTOXIC PROTECTIVE POTENTIAL OF PLANT SECONDARY METABOLITES

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Reg.No.U74120 MH2013 PTC 251205
Harshwardhan Publication Pvt.Ltd.
At.Post.Limbaganesh,Tq.Dist.Beed
Pin-431126 (Maharashtra) Cell:07588057695,09850203295
harshwardhanpubli@gmail.com, vidyawarta@gmail.com
All Types Educational & Reference Book Publisher & Distributors www.vidyawarta.com

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❖ **Publisher :**
Harshwardhan Publication Pvt.Ltd.
Limbaganesh, Dist. Beed (Maharashtra)
Pin-431126, vidyawarta@gmail.com

❖ **Printed by :**
Harshwardhan Publication Pvt.Ltd.
Limbaganesh, Dist. Beed, Pin-431126
www.vidyawarta.com

❖ **Page design & Cover :**
Shaikh Jahuroddin,Parli-V

❖ **Edition: June 2020**

ISBN 978-93-90284-01-6

❖ **Price : 149/-**

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Dedicated to
my beloved family

PREFACE

The use of many chemotherapy and other drugs is often accompanied by a great deal of adverse side effects. Increased exposure to industrial effluents, environmental pollutants and other chemicals also pose a threat to human health, as many of these chemicals are genotoxic and cause clastogenic and mutagenic changes in the genome, which can result in carcinogenesis. This imposes a great necessity to search for natural compounds with therapeutic potential as well as chemopreventive abilities to diminish the adverse effects of synthetic drugs or environmental pollutants. Natural compounds can be employed as adjuvants in therapeutic strategies or may be included as dietary supplements to strengthen the innate defense mechanisms, to counter the genotoxins. A great number of plants and their active constituents exhibit medicinal properties. Natural compounds, especially those which have been in use in ethno-pharmacological systems since a long time, are generally assumed to be safe. But it is vital to validate the safety of these substances in terms of genotoxic and clastogenic effects.

This book gives an insight into the genotoxic and antigenotoxic properties of plant secondary metabolite in general, with emphasis on two selected active constituents Apocynin and Diosgenin. These two compounds have displayed valuable therapeutic potential in different experimental models, in addition to being used in traditional medicinal systems like Ayurveda. But the information regarding the genotoxic/antigenotoxic nature of these two compounds was scarce. Hence, they were chosen for the research work, on which this book is based upon.

The author, Dr.G.Hema, has more than 14years of teaching and research experience as Assistant professor and Head of the Department of Biotechnology, at Maharani's Science College for Women, Bangalore. The author holds an excellent academic record including qualifying in national level exams like the ICAR entrance

and CSIR-UGC JRF and NET. She has had the privilege of working in research projects at prestigious institutes like the Institute of Microbial Technology, Chandigarh and the Tamil Nadu Agricultural University, Coimbatore. The author has obtained her Ph.D degree in Biotechnology from the Jawaharlal Nehru technological University Anantapur, A.P. She brings out her doctoral research work after editing, in the form of this book, to facilitate easy access and use by scientific fraternity and general public. The author is currently working on research areas pertaining to Microbial degradation of environmental pollutants & Antiviral properties of plant bioactive extracts.

The book is presented in six chapters. The chapter-1 is an introduction to various concepts involved in the study, like the effects of genotoxicity, the need to overcome such effects, importance of plant based and other natural substances in biochemical processes and the methodology that can be adopted to evaluate these substances.

Chapter-2 comprises a deep review of literature with respect to the mechanisms underlying the genotoxic effects of substances, available experimental methods to assess genotoxic end points, pharmacological potential of natural substances and the underlying molecular mechanisms.

Chapter-3 is a description of all the materials used in the present study and the experimental procedures. The results of all the experimental studies carried out are compiled into chapter-4.

In chapter-5, a detailed discussion of the antigenotoxic and anticytotoxic effects displayed by apocynin and diosgenin is done. The different mechanisms that have been proposed for such effects of other natural compounds are mentioned since they would form a basis for understanding the protective effects displayed by the compounds of interest in the current study.

The complete study has been summarized in chapter-6. This is followed by a list of references that have been reviewed for the book.

❑ CONTENTS ❑

CHAPTER - 1

1.1 General Introduction

The integrity of genome is the most important factor deciding the fate of a cell. DNA, the heritable macromolecule which constitutes the genome, is highly susceptible to damage by different chemical and physical agents, and this may affect its integrity and functionality. Prevention of damage as well as repair of the damages to the genome is highly essential to ensure the viability of healthy cells and continue their lineage. Cells are inherently provided with stringently controlled biochemical pathways to detect the modifications in DNA and to repair them, so as to ensure the fidelity of DNA replication and segregation of chromosomes correctly to the next generation of cells. Unrepaired damages lead to mutations and other abnormalities [1]which may further lead to carcinogenesis.

These carcinogens are majorly grouped into genotoxic and epigenetic (non-genotoxic). Genotoxic agents are carcinogens that usually are capable of causing direct damage to genetic material,

which in turn can lead to mutagenic and/or clastogenic alterations [2].Genotoxicity is a broad phenomenon that covers the ability of a substance, not only to alter the DNA, but also other cellular components that regulate the conformity of the genome and functionality and behaviour of chromosomes within the cell, for instance, the mitotic apparatus and topoisomerase enzymes. Genetic toxicology is the study of such agents, their mechanism of action and the consequences [3].

Humans persistently are exposed to quite a lot of environmental pollutants, industrial chemicals and effluents, heavy metals, cytotoxic drugs, dyes and colours used for different purposes that can lead to the development of different kinds of cancer [4–7]. Under conditions of extreme stress, the cellular biological machinery fails to overcome the genotoxic injuries caused by different agents, leading to malignancy. Toxicity of substances is manifested in diverse forms like DNA damage, Chromosomal aberrations, formation of micronuclei, cell death or abnormal cell growth leading to tumour formation. Contact with such agents is often inescapable and creates a huge menace to human health.

The human body is innately equipped with molecular detoxification systems to defend against these toxins [8, 9]. But increased or repeated exposure to hazardous chemicals can lead to mutagenic events, as in the case of cancer chemotherapy. Cancer is caused by the genomic alterations leading to the accumulation of the abnormal cells which undergo uncontrolled cell division and can disperse to other tissues of the organism. Though chemotherapy remains a principle agent for the treatment of cancer and is expected to have the ability to differentiate between a normal and cancerous cell, toxicity issues to normal cells remain the major obstacles in its clinical use [1, 10, 11]. Fortifying the inherent defences with exogenous antigenotoxic agents becomes essential to prevent neoplasia. A viable approach in such situations is to help the

individual to defend against mutagens and carcinogens by dietary supplementation with chemopreventive and chemoprotective agents [12, 13].

Several mechanisms of direct and indirect genotoxicity have been identified [14]. Oxidative stress is known to generate a multiplicity of DNA damage [15]. A shift in the prooxidant and antioxidant balance of the biological environment in favour of prooxidant state leads to oxidative stress. This occurs when cells are incessantly exposed to reactive oxygen species (ROS). There are contributed by both endogenous sources of normal metabolism and exogenous sources such as environmental pollutants, chemotherapeutic drugs and industrial chemicals. Oxidative DNA damage, combined with a malfunction of cellular DNA repair mechanisms, is extensively associated with carcinogenesis and other pathobiological conditions [16]. ROS may induce single- and double-stranded breaks, DNA crosslinks and base modifications, all of which are concerned in initiation and promotion of tumours. Under conditions of extreme oxidative stress induced by diverse sources, for instance chemotherapy, to tackle the genotoxic damage, use of antimutagens and antigenotoxins, from natural sources is a well-founded approach [17, 18].

It is crucial to investigate the genotoxicity of a compound, in the milieu of understanding its mechanism of carcinogenicity. Genotoxic carcinogens propound a larger peril to humans than non-genotoxic carcinogens [19]. Moreover, it is inevitable to understand the antigenotoxic properties of compounds, to be able to deploy them in strategies of fighting cancer. In this context, it is also of significance that the cytotoxic and anticytotoxic nature of substances is evaluated, to make certain their safety for therapeutic purposes. Such investigation of antimutagenic capabilities of natural products can lead to development of potential chemotherapeutic, chemopreventive agents.

Plants produce secondary metabolites in response to environmental stimuli. A vast number of these phytochemicals have been isolated and their benefits to human health have been documented. Crude extracts and biologically active compounds isolated from natural substances, especially those which are in use in ethnic medicinal systems, can be prolific sources of new drugs, with priceless application in treatment of various pathological conditions [20]. Topical health concerns have paved enormous deal of attention towards the natural antioxidants in plants for their medicinal and biological activities taking into consideration, the numerous shortcomings of synthetic compounds for human population [21]. Natural substances play a significant role in multiple biological mechanisms, contributing to their antigenotoxic and anticarcinogenic effects. The majority of these are known to wield their effects either by quenching ROS or by stimulating cellular defences. Plants are precious sources of such biologically active molecules. Research, across the world has been focussing on exploring the antigenotoxic, anticytotoxic and anticancer potentials of plant compounds. At the same time, it is highly important to evaluate their efficacy and safety as putative inhibitors, for clinical relevance. It also is important to understand the mechanisms involved therein [22].

The plant secondary metabolites, grouped as phenols and phenolic compounds have been shown to hold abundant therapeutic properties [23–25]. Phenolic compounds act as inhibitors of free radical production and also as free radical scavengers. They can also indirectly act by modulating the activity of enzymes with antioxidant, detoxifying and repairing functions [13]. Several phenolic compounds are being studied for their possible use as antigenotoxic agents [13, 26–29]. Apocynin is one such phenol, renowned for inhibition of the complex NADPH-oxidase and is endowed with myriad applications in the treatment of inflammatory and oxidative disorders like arthritis, asthma and hypertension [9],

which imply its potential use in treatment of several pathological conditions. Reports on the genotoxic and antigenotoxic abilities of apocynin are scarce. In view of the vast potential of this compound as a therapeutic agent, we chose to study the genotoxic and antigenotoxic properties, as well as the cytotoxic and anticytotoxic nature, of this compound.

Another major group of plant secondary metabolites with extensive biological and therapeutic performance are saponins. Saponins are the principle constituents in many herbal medicines and recognised for the health benefits offered by foods like soya [30]. Diosgenin is a steroidal saponin present in abundance in legumes and yams. It is extensively utilised in the pharmaceutical industry as starting point of various synthetic steroidal drugs. The antioxidant nature of this compound is well established [31]. The role of diosgenin and plant extracts containing diosgenin as an active principle, as chemopreventive or chemotherapeutic agents is being elaborately studied [32,33]. Efforts are being taken towards understanding the favourable role of diosgenin in case of metabolic diseases like hypercholesterolemia, obesity, dyslipidemia, inflammation, and diabetes [34]. An essential requirement in this process would be the understanding of genotoxic and antigenotoxic influences of diosgenin, on which very limited information is available. In this context, diosgenin was chosen as a part of this research work. In the present study, we investigated these two selected active plant constituents, apocynin and diosgenin, for their genotoxic and antigenotoxic properties. This could provide valuable leads in search of compounds that can reduce the occurrence of clastogenic and mutagenic alterations and consequent degenerative diseases.

Assessment of genotoxic and antigenotoxic nature of substances can be done by a series of *in vitro* and *in vivo* assay systems that became established over a period a time, as suitable indicators, under various international guide lines. A number of

experimental systems are also available for ascertaining the cytotoxic and anticytotoxic nature of substance. The experimental methodology for the current study was based on the recommendations in the various internationally accepted guidelines, chosen after reviewing a number of research reports in this area of science by researchers from different countries. Assays based on both animal and plant systems can be employed for a deeper understanding of the genotoxic nature of the selected plant compounds [35].

With this background, the current research work was proposed and carried out to explore the genotoxic/ antigenotoxic properties of apocynin and diosgenin against cyclophosphamide using the mouse bone marrow micronucleation test, measuring the haematological and oxidative stress parameters. The compatibility of apocynin and diosgenin with biological systems was further evaluated by testing their cytotoxic and anticytotoxic properties in cell culture systems. We also employed the *Allium* root tip meristem analysis, haemolytic and DNA fragmentation assay for a broad interpretation of the genotoxic and antigenotoxic properties of apocynin.

The aim of the present investigation was to evaluate potential genotoxic and antigenotoxic effects of apocynin and diosgenin against chromosomal and genome damage induced by the chemotherapeutic agents in living cells and tissues, using *in vitro* and *in vivo* assays.

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CHAPTER - 2

2.1 Genotoxicity and its Principal Mechanisms

Genetic toxicology is gaining importance among the scientific fraternity across the globe since it affects not only the individual but also his progeny. As an effort to tackle this situation, attention to mutation research has increased and a significant pool of evidence has been accumulated towards the mutagenic potential of medicinal agents, environmental pollutants and industrial chemicals. Genotoxicity tests are a critical component of safety evaluation of drugs and chemicals like pesticides, food additives and packaging material. To obtain valid data on the mutagenic and carcinogenic properties of a chemical, information regarding the genotoxic effects of the chemical at different levels namely, the DNA, the chromosomes and the cellular machinery like spindle apparatus, necessary for chromosome segregation, is very much essential[36].

Genotoxicity is the ability of a chemical agent or radiation to cause damage to the genetic information within a cell by

chemically interacting with DNA and/or non-DNA targets [37]. Such damage in germ cells affects reproduction or lead to heritable mutations. In case of somatic cells, it may lead to malignancy. All mutagens are genotoxic, while all genotoxins are not [37, 38].

Several mechanisms of genotoxicity have been proposed which can be broadly grouped as direct and indirect (Figure 2.1.1). Direct damage to DNA involves formation of oxidative DNA adducts, DNA strand breaks, nitrogen base (e.g., formation of 8-hydroxydeoxyguanosine adducts) or deoxyribose modifications, and cross-links [14, 15,39]. Continual DNA damage may result in genomic instability, replication errors, arrest or induction of transcription, and signal transduction pathways, which eventually lead to carcinogenesis [15].

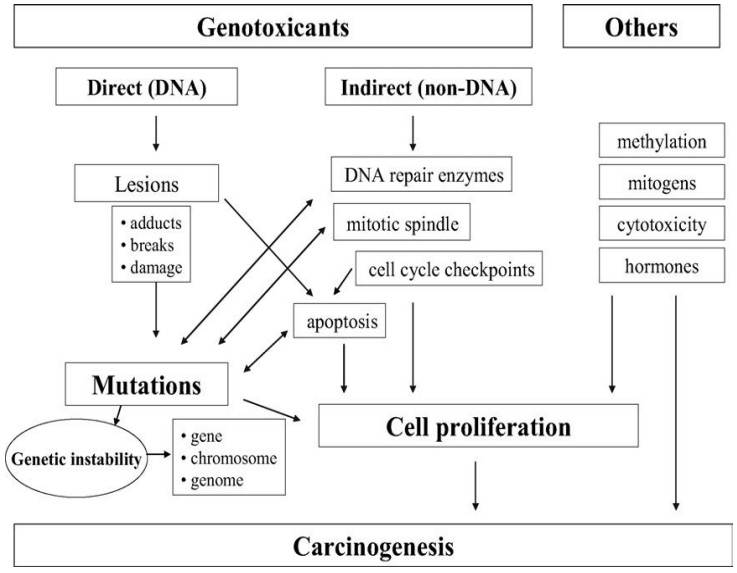


Figure 2.1.1 Different mechanisms of genotoxicity
(adapted from Kirsch-Volders *et al*., 2003 [14])

Genotoxicity can also occur through indirect mechanisms where in the genotoxic agent interacts with non-DNA targets, leading

to formation of protein adducts, oxidative stress, inflammation and abnormal signalling responses [40]. Target proteins include repair enzymes, DNA and RNA polymerases or other enzymes involved in DNA replication, factors involved in apoptosis (e.g.p53, bax, bcl-2), proteins that regulate cell cycle (e.g. p53, cyclins), and proteins such as glutathione that defend against oxidative damage [7,14, 41–43].

Genotoxins cause oxidative stress in the cellular environment and generate free radicals. Free radical-initiated damage to biomembranes results in peroxidation of lipids, which in turn causes production of such as reactive electrophiles like epoxides and aldehydes like Malondiadlehyde (MDA) [15]. Reactive oxygen species (ROS) can also induce cell proliferation and apoptosis [44, 45], alter gene expression by altering signalling pathways (e.g. cAMP-mediated cascades, calcium-calmodulin pathways), and activation of transcription factors. Different genotoxic agents employ different mechanisms of causing damage. They also may employ more than one mechanism and may vary with the type of tissue/ cells [2, 5, 46,47].

Genotoxic endpoints like incidence of micronuclei, chromosomal aberrations and DNA fragmentation are well known markers of genotoxicity and diminution in the occurrence of these genotoxic endpoints is indication of the antigenotoxicity of a particular compound [48].

2.2 Genotoxicity and Cytotoxicity Assays

2.2.1 Genotoxicity assays

Several *in vivo* and *in vitro* assay systems for assessing the genotoxic/ antigenotoxic and cytotoxic/cytoprotective properties of substances have been developed. A test should be sensitive as well as specific [49]. Due to the diversity of the biomarkers for genotoxicity and/or mutagenicity of a compound, it is essential that

multiple tests need to be conducted to evaluate a test substance. Different organisations and advisory bodies have formulated guidelines for such evaluation. The Organisation For Economic Cooperation and Development (OECD) [50] recommends that the battery of tests include short and long term tests, as well as *in vitro* and *in vivo*.

Assays are designed based on diverse endpoints of genotoxicity such as point mutations, deletions, insertions, gene amplifications, micronuclei (Mn), chromosomal aberrations (CA) and sister chromatid exchanges (SCE). The various test systems widely adopted by the scientific community for genotoxic assessment of compounds are described briefly here.

2.2.1.1 Bacterial reverse mutation test /Ames test (OECD TG 471)

This assay is based on identification of reverse mutations in *Salmonella typhimurium* strains with defined mutations in the histidine locus and hence, auxotrophic for histidine. Bacteria are exposed to the test compound, and the revertants, able to survive in the absence of histidine are selected. Strains of *Escherichia coli* are also employed [51, 52].

2.2.1.2 In vitro mammalian chromosome aberration test (OECD TG 473)

The test identifies substances that can cause structural chromosome aberrations and numerical changes such as polyploidy and duplication in cells. It employs cultures of established mammalian cell lines or primary cell cultures. Most compounds that are positive in this test are mammalian carcinogens [38, 53].

2.2.1.3 Mammalian erythrocyte micronucleus test (OECD TG 474)

Erythrocytes are sampled from the bone marrow and/or

peripheral blood cells of experimental animals (mice or rats), for analysis of damage to the chromosomes or the mitotic apparatus caused due to test compounds. The cytogenetic damage is depicted as micronuclei formed by lagging chromosome fragments or whole chromosomes [54].

2.2.1.4 Mammalian bone marrow chromosome aberration Test (OECD TG 475)

Structural chromosome aberrations induced in bone marrow cells of laboratory animals, usually rodents, treated with the test substance. Sometimes animals may be also treated with a metaphase-arresting agent. Bone marrow cells are collected, stained and analysed for chromosome aberrations [55].

2.2.1.5 In vitro mammalian cell gene mutation test (OECD TG 476) or the mouse lymphoma assay (MLA) assay

It detects mutations involving the thymidine kinase (Tk) locus in mutant mouse lymphoma cells. Other Suitable cell lines include the CHO and TK6 human lymphoblastoid cells. Mutations in hypoxanthine-guanine phosphoribosyl transferase (HPRT), and xanthine-guanine phosphoribosyl transferase (XPRT) loci are also commonly identified endpoints [56].

2.2.1.6 In vitro sister chromatid exchange assay in mammalian cells (OECD TG 479)

SCEs are interchanges of DNA replication products at homologous loci between the sister chromatids of a replicating chromosome. The cells *in vitro* are exposed to the test chemical and grown for two generations in BrdU-containing medium, for differential labelling of the sister chromatids. Then they are subjected to treatment with a spindle inhibitor like colchicine to arrest the cells in a metaphase-like stage of mitosis (c-metaphase) and enumerated for SCE's [56, 57].

2.2.1.7 In vitro mammalian cell micronuclues test (OECD TG 487)

Clastogenic (structural) and aneugenic (numerical) changes induced by test substances in cells that have undergone cell division during or after exposure to it are detected. Inclusion of the actin polymerisation inhibitor cytochalasin B, allows for the identification and analysis of Mn in cells that have completed one cycle of mitosis, because such cells are binucleate. The use of protocols without cytokinesis block is also allowed provided there is evidence that the cell population analysed has undergone mitosis [58].

2.2.1.8 Comet assay/ single cell electrophoresis test

This is single cell cytogenetic approach for detection of DNA crosslinking, single strand breaks and alkali-labile sites. This highly sensitive assay detects damage at individual cell level, even at alkaline pH and also provides for very specific damage detection using enzymes and antibodies [59].

2.2.1.9 Plant assays for assessing the genotoxicity

Higher plant genetic bioassays such as *Allium* root tip meristem analysis test and the Barley chlorophyll mutation assay are highly recommended to be relevant for the detection of chromosome aberrations and gene mutations induced by test substances. Other plants that are frequently used are *Crepis capillaris, Lycopersicon esculentum, Hordeum vulgare, Pisum sativum, Tradescantia, V. faba* and *Zea mays* [35, 60, 61]

2.2.2 Cytotoxicity assays

Cytotoxicity of substances plays a major factor in a number of pathological processes, like inflammation and carcinogenesis. Evaluating the cytotoxic nature of substances is essential in understanding the mechanisms of action of those substances on living cells and tissues [7,26, 62–65]. Several cytotoxicity assays based on animal cell culture systems, which allow measurement of

cell growth inhibition in response to chemicals tested, in a rapid and precise manner, are widely used in preliminary studies. They enable the select the suitable concentrations of the tested compound for further experiments and are also used in alternative *in vitro* methods for regulatory purposes. Assays are designed based on the measurement of various cytotoxicity biomarkers like cell membrane integrity, mitochondrial injury, lysosomal dysfunction, cell protein content, incorporation of radioisotopes, colorimetric and luminescence measurement tests.

Biological membranes can be damaged by cell disaggregation, cell separation or freeze –thaw process. This lose of integrity can be determined by uptake of dyes to which normally viable cells are impermeable (e.g. naphthalene black, erythrosine, trypan blue) or by the release of dyes normally taken in and retained by intact cells (e.g. neutral red, diacetyl fluorescein). Researchers across the world have evaluated and suggested the use of various *in vitro* assays in toxicology [66]–[68]. The most widely adopted testing procedures are given below.

2.2.2.1 Trypan blue (TB) staining

Viable or intact cells are impermeable to dyes such as trypan blue, nigrosin green, naphthalene black, and erythrocin B. As such, cells dead due to damaged plasma membrane allow entry of the stain, whereas live cells with intact plasma membrane do not stain. The number of intact (unstained) cells is a direct measure of the cell viability in the preparation.

2.2.2.2 Neutral red (NR) uptake test

Living cells stain with neutral red by active endocytosis through the cell membrane and its storage into lysosomes and Golgi apparatus. The number of stained cells gives the number of viable cells.

2.2.2.3 MTT, MTS and XTT assays

These assays are based on measuring the activity of mitochondrial dehydrogenases as an indicator of cell viability and cell number. A population of cells in culture are exposed to the tested compound drug during the log phase. The test compound is then removed and cells are allowed to proliferate for 2-3 population doubling times. They are then incubated with the tetrazolium salt substrate to allow its conversion into a coloured formazan product, by mitochondrial dehydrogenases. The commonly used tetrazolium compounds include MTT, MTS, XTT, and WST1. These are basically of two categories:

1. Positively charged salts like MTT and capable of penetration into viable eukaryotic cells
2. Negatively charged salts like MTS, XTT and WST1, which do not readily penetrate cells.

The number of surviving cells is directly proportional to the spectrophotometric measurement of the concentration of the colour intensity.

2.2.2.4 Sulforhodamine B (SR-B) assay

The SR-B dye binds to the proteins of cells fixed with Trichloroacetic acid. The amount of bound dye is a measure of cellular protein which indirectly determines the total number of cells.

2.3 Description of Genetic Toxicity Tests

2.3.1 Mammalian bone marrow micronucleus assay

The bone marrow micronucleus (Mn) assay system has been widely used for assessing genotoxicity and cytotoxicity [69]. Micronucleus is described as a small extra nuclear chromatin body originating from an acentric fragment or whole chromosome lost from the metaphase plate. They are also known as Howell–Jolly bodies, since their identification by the American scientist, William Howell, and Justin Jolly, a Frenchman. They appear as distinct membrane bound chromatin bodies in the cytoplasm of eukaryotic

cells exposed to genotoxic agents. The significance of this technique was emphasised by several studies as a consistent method for measuring of both chromosome breakage and chromosome loss caused by cytotoxic agents [41, 70]. Most preferred experimental animals are mice. Other rodents such as rats and hamsters are also used. Most suitable tissue for enumerating the micronuclei is the bone marrow as it is the major haematopoietic tissue, has rapidly dividing cells and is well-vascularized, in mature animals. Exposure to a test chemical during proliferation of erythropoietic cells can lead to chromosome breakage and damage to the mitotic apparatus. These broken chromosome fragments or lagging chromosomes form micronuclei. When the erythroblast develops into a an erythrocyte, the main nucleus is extruded and the micronuclei are left in the cytoplasm and hence, well visualised by staining [71]. Moreover, the PCE still contains RNA, and stains blue grey with Giemsa, where as mature, haemoglobin-containing erythrocytes, stain orange with Giemsa. This aspect enables identification of the cells with micronuclei induced by the test substance. It is important to collect PCEs from the bone marrow or peripheral blood before their differentiation to mature erythrocytes. The induction of micronuclei may lead to cellular lethality and mutation, leading to a lower number of PCE. Erythropoietic safety assessment in new compounds is vital in drug discovery and development. Both structural (clastogenic) and numerical (aneugenic) chromosome changes can be detected. Since it is relatively simple and at the same time shows high sensitivity, the *in vivo* rodent erythrocyte micronucleus assay has gained extensive acceptance [54].

Mechanisms of micronuclei formation

Micronuclei harbouring chromatin bits are products of breakage of double-stranded DNA molecule, SSBs that get converted into DSBs following replication, or arrest of DNA synthesis. Fusion of two broken chromosomes could result in a

dicentric chromosome and an acentric piece of chromatin. These acentric fragments in anaphase lag behind to form micronuclei. Mn with whole chromosomes appear due to defects in the genes of segregation machinery, spindle apparatus, mechanical or chemical disruption of centromeric DNA [72, 73]. Mn may also be generated through the breakage-fusion-bridge (BFB) cycles [74]. Other modes of Mn formation include

i. Acentric chromosome/chromatid fragments resulting from SSB or DSB
ii. Whole chromosomes/ chromatids that lag behind in anaphase
iii. Depolymerization of tubulin
iv. Defects in centromeric DNA, proteins or assembly
v. Late replication, peripheral location in the nucleus and epigenetic modifications of histones
vi. Formation of nucleoplasmic bridges
vii. Asymmetrical repair of two broken chromosomes producing a dicentric chromosome and an acentric fragment
viii. Dicentric chromosomes formed by telomere end fusions.
ix. Breakage-fusion-bridge cycles leading to DNA amplification and its selective elimination via nuclear buds [73].

2.3.2 Estimation of oxidative stress bio-markers in liver

2.3.2.1 Estimation of lipid peroxidation in the liver:

The metabolism of most endogenous compounds as well as exogenous substances in the human or animal body is primarily centred in the liver. Reactive oxygen species (ROS) or free radicals are generated during the metabolic processes in the liver tissues. They play an important role in modulating normal cellular functions, as secondary messengers. An imbalance between the production of ROS and their removal leads to a condition commonly termed as oxidative stress [75]. ROS can adversely affect nucleic acids, lipids, and proteins leading to disturbance in cellular homeostasis.

ROS are relatively short-lived and the damage caused is predominantly local. But, they initiate oxidative deterioration of lipids in the cell, leading to production of aldehyde by-products. These secondary oxidative species have longer half-lives and can diffuse to distant intra- and extra -cellular targets, causing further oxidative damage. The production of ROS is amplified due to impairment of mitochondrial function by the aldehyde by-products [76]. Lipid peroxidation is a direct indication of oxidative stress. It is involved in aging and several pathological disorders [77]. The estimation of lipid peroxidation by using the primary oxidative species is rendered impractical by their labile, short-lived nature. As a result, detection of LPO is mostly based on indirect methods based on analysis of secondary products like MDA [78].

The quantification of MDA in tissue or cell homogenates is widely done by the method of Okhawa *et al*., 1979 [77]. MDA is a volatile by product of enzymatic oxygenation of arachidonic acid and other lipids. It is a short-chain, low-molecular-weight ($C_3H_40_2$; formula weight = 72.07), 1,3- dicarbonyl compound with a pKa of 4.46. This is a sensitive assay involving the reaction of Thiobarbituric acid (TBA) with Thiobarbituric acid reactive substance (TBARS or MDA) during aerobic incubation with tissue homogenates, forming a fluorescent red product (Figure 2.3.5) [78]. This method has been widely employed by researchers to quantify lipid peroxidation in a diversity of chemical and biological matrices as a biomarker of oxidative stress. The oxidant/anti-oxidant behaviour of substances subsequently is an indication of genotoxic/ antigenotoxic abilities of the compounds being investigated [27, 79–83].

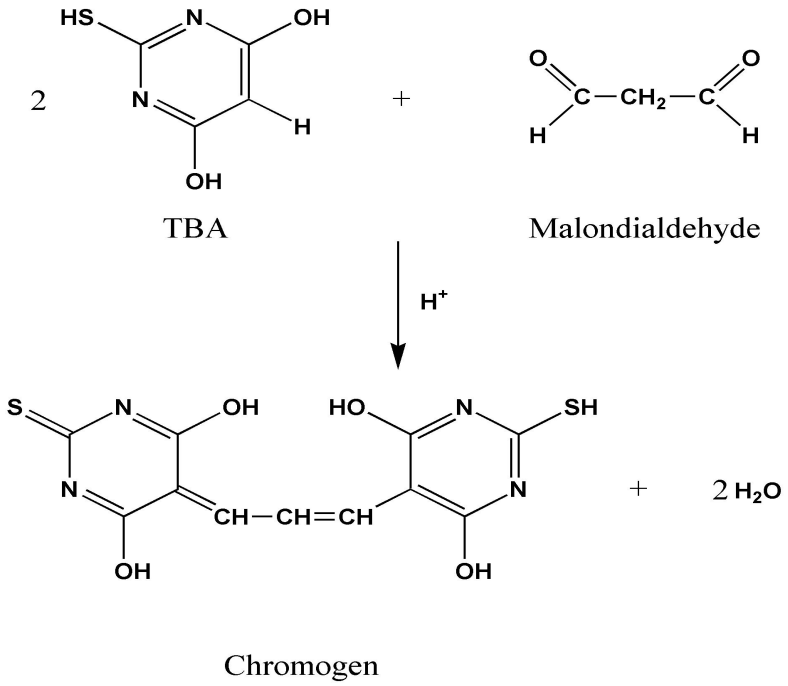


Figure 2.3.5 Chromogenic reaction of MDA with TBA

2.3.2.2 GSH estimation

An antioxidant is a substance that significantly lowers or inhibits oxidation of a substrate even when present at low concentrations, compared to that of the oxidizable substrate [84]. Reduced glutathione (GSH), is the most prevalent intracellular antioxidant. It is a low molecular weight soluble thiol synthesized in cells. GSH is a tripeptide, γ-L-glutamyl-L-cysteinyl-glycine. The sulfhydryl group (-SH) present in cysteine is crucial for the activity of GSH across a variety of metabolic processes, like synthesis of proteins and nucleic acids, transport of cellular molecules, enzyme activity, peroxide removal and cell-cycle regulation [85].

Cytosolic concentration of GSH is in the range of 1–10

mM. The hepatocytes export GSH, and have a high concentration of about 10 mM. It regulates apoptosis in mitochondria and cellular division in the nucleus. The ratio of reduced and oxidized forms of glutathione (GSH/GSSG) is an indicator of oxidative stress. Detoxification of H_20_2 and lipid peroxides by GSH is catalysed by the enzyme GSH reductase (GSHPx) using NAD(P)H as electron donor. Under normal conditions, above 98% of cellular glutathione exists as GSH. GSH acts as proton donor to membrane lipids against oxidant attacks. It is a cofactor for enzymes, such as glutathione peroxidase and transferase, involved in cellular detoxification. It also is involved in onverting back other cellular antioxidants like vitC and vitE to their active forms and regulation of several transcription factors such as AP1,NFκB, and Sp1[2, 44, 85].

Due to this immensely important role of GSH in preservation of cellular redox balance, GSH has gained much importance in chemotherapy [86]. Measurement of GSH is based on its reaction with Dithionitrobenzoic acid (DTNB). DTNB reacts with GSH forming a conjugate and TNB anion that are quantified by absorbance.

2.3.3 Allium cepa root meristem cell analysis

The *Allium cepa* test has been widely accepted as a standard cytogenetic system for evaluation of potential genotoxic and cytotoxic effects of environmental pollutants as well as other synthetic and natural compounds of interest [87–90]. This plant is of choice due to low raising costs, ease of handling, rapid execution, high sensitivity and appropriate chromosomal features. It has large chromosomes (2n = 16), facilitating good and convenient microscopic observation of chromosome damages and aneuploidy [91]. Available literature shows the data obtained from this system can be well correlated with that from prokaryotic and animal systems [29, 92, 93].

The two parameters that are evaluated as biomarkers of genotoxicity of substances, in the root tip meristems are mitotic index (MI) and the frequency of chromosomal aberrations (fCA). Mitotic index is obtained by counting all stages of mitotically active cells per 1000 cells. Lowering of mitotic index is an indicator of the mitodepressive effect of test substance [94, 95]. The mitotic index is given by

$$MI = \frac{n_d}{n_t} \times 100\%$$

[n_d - number of dividing cells and n_t –total number of cells].

The number of cells showing mitotic abnormalities like lagging chromosomes, spindle abnormalities, adherent nucleus, anaphasic bridges and broken chromosomes, in anaphase or telophase, are counted per 1000 cells, in each root tip, by scanning the slides from right to left, up and down. The frequency of chromosomal aberrations is calculated as

$$fCA = \frac{n_a}{n_t} \times 100\%$$

[n_a is the number of aberrant cells]

2.3.4 MTT Assay

The MTT (3(4,5dimethylthiazol2yl) 2,5diphenyltetrazolium bromide) tetrazolium reduction assay is a high throughput screening (HTS) [96] assay developed for a 96well format [97]. It has been widely adopted in academic and research labs [98–102]. Only viable cells with active mitochondrial functioning convert yellow MTT into purple coloured formazan. The intensity of colour formation is directly proportional to the number of viable cells. The absorbance is measured at 570 -595 nm [103] in a plate reading spectrophotometer. The MTT- formazan product is insoluble and precipitates in the cells and culture medium. This needs to be

solubilized before reading the absorbance. A dose-response curve is prepared and used to calculate the IC50 value of the test agent [104]. Sensitivity of the MTT assay is influenced by factors like the physiological state of cells and variations in dehydrogenase activity.

% growth inhibition = 100 - % Cell viability

Cell viability (%) = Mean OD/Control OD x 100

2.3.5 DNA fragmentation assay

Oxidative DNA damage is considered as carcinogenic and can actively lead to occurrence of pathological conditions like cancer and aging. Natural compounds offer protection against oxidative DNA damage by directly scavenging the free radicals or by reducing their production when incubated simultaneously with the genotoxic and antigenotoxic agents. In case of pretreatment, they may exert effects such as increasing the level of cellular enzymatic and non-enzymatic antioxidants, inhibition of phase I bioactivating enzymes and/or induction of phase II detoxifying enzymes [19,105]. DNA fragmentation is also a hall mark of apoptotic cell death. Several carcinogenic and chemotherapeutic agents, such as methylprednisolone, 4-hydroxytamoxifen, methotrexate, 5-fluorouracil, cisplatin, y-irradiation, and nitrogen mustard, are known to induce apoptosis. During apoptosis, caspase 3 activates ICAD to CAD by cleavage. CAD in turn cleaves the DNA at the linker sites between the nucleosomes. The degree of DNA damage is evaluated by extraction of DNA from the treated cells or tissues and resolving it on an agarose gel or by performing comet assay, along with DNA from suitable controls. The antigenotoxic effects of several natural substances has been confirmed by their ability to prevent DNA fragmentation *D. speciosum* extracts wield protection against 4NQO induced genotoxicity, demonstrated by reduced DNA fragmentation, acting as a desmutagenic agent [106]. In yet another study, bixin, was found to decrease DNA fragmentation in cisplatin-treated PC12 cells [107].

2.3.6 Haemolysis assay

Haemolysis is the lysis of erythrocytes induced by the effect of varied substances like contact toxins, metal ions and drugs, on blood. It releases iron and other components into the circulatory system and also leads to anaemia. Particularly during prolonged contact, haemolysis may cause adverse effects [108,109]. The biological haemolytic or antihaemolytic properties of compounds need to be assessed as an indication of their biological safety and protective efficiency [110–112].

2.4 Drugs and their Genotoxicity

Most of the chemotherapy agents cause DNA damage leading to growth inhibition and apoptosis. But most of the these are non-selective in their action with significant cytotoxicity and genotoxicity to normal cells. Incidence of secondary malignancies is a major constraint of chemotherapy [113]. Therefore it is vital to include agents that can avoid these side effects in chemotherapy regimen.

Cyclophosphamide (CP) is an alkylating chemotherapy drug, widely used for treating malignant and non-malignant disorders. The mutagenicity and genotoxicity of cyclophosphamide has been extensively studied *in vitro* and *in vivo* [114, 115]. The cytotoxic metabolites of this alkylating agent are acrolein and phosphoramide mustards, capable of causing DNA crosslinking and strand lesions. It caused significantly higher frequency of MnPCEs in mice and other experimental organisms compared with controls in several studies [65, 80, 116].

Cisplatin has genotoxic effects like chromosomal aberrations, formation of Mn and granular chromatin condensation in cultured mammalian cells and in bone marrow and other types of cells and also sperm head abnormalities in experimental animals [86]. The induction of a Mn by Cycloplatum, a chemotherapy drug

in vivo and *in vitro* indicates a potential risk for patients [117]. Studies by Vijaylaxmi *et al*., [118] showed induction of transplacental micronuclei in mice by Carboplatin, a widely used platinum anticancer drug. Oxaliplatin also causes CAs and SCEs in human lymphocytes [119].

Melphalan and Mitoxantrone are also anthracycline antibiotics used in cancer therapy capable of inducing genotoxic effects in human lymphocytes *in vitro* in a dose-dependent manner [120]. Doxorubicin, another anthracyclin antibiotic, used in the treatment of leukaemia, lymphoma, solid tumours of the breast and ovaries has dose-limiting, cardiotoxic side effects caused by increased ROS production and genotoxic effects induced by inhibition of the enzyme topoisomerase II [121]. Investigations by YurtCu *et al*., [122] demonstrated significant genotoxic and cytotoxic effects of Doxorubicin and Silymarin, individually or in combination on HepG2 cells. Another semisynthetic anthracycline antibiotic Epirubicin, also causes micronucleation in mice bone marrow [123]. Direct strand breaks, oxidation and methylation of the DNA bases can underlie the DNA-damaging effect of anticancer drugs [124].

Taxanes are drugs effective against tumours in different organs, lead to development of nonfunctional microtubule bundles [125]. 5-Fluorouracil (5-FU) another widely used antimetabolite anti-neoplastic drug in cancer therapy, caused DNA damage in the liver and blood cells, and micronuclei in erythrocytes of zebra fish[126].

A vast number of drugs used to treat pathological conditions other than cancer, also possess genotoxic and cytotoxic abilities. A compendium on the genotoxicity information of more than four hundred structurally diverse pharmaceutical products by Snyder and Green [10] indicates that 28.7% of the studied compounds

had positive results in at least one of the genotoxicity assays. Several marketed bronchodilators and asthmatics had tested positive for genotoxic effects [11]. A review by Brambille *et al*., [127] indicates that many antibacterial, antimalarial, antiviral and antifungal drugs showed genotoxic and carcinogenic abilities *in vitro*.

2.5 Significance of Antigenotoxic Agents

2.5.1 Role of ROS and antioxidants in genotoxicity

ROS are formed as a result of normal metabolism, but at high concentrations, they adversely affect cell components. A shift in the cellular redox balance towards oxidants is termed "oxidative stress." This can occur either due to exhaustion of antioxidants or build up of ROS. Various forms of reactive oxygen species are singlet oxygen ($_1O^2$), superoxide anion (O^{2-}), peroxyl radicals (ROO*), hydrogen peroxide (H_2O_2), and hydroxyl radicals (*OH). O^{2-}combines with NO to yield peroxynitrite anion (ONOO") and peroxynitrous acid, which are secondary oxidizing entities. This reduces NO bioavailability and is termed as nitrosative stress [16].

A sufficient level of intracellular antioxidants is crucial for maintaining normal cellular function. The inherent antioxidant defence system of human body is composed of several endogenous antioxidants, which act by different mechanisms such as scavenging of oxidative species, reduce them to non-toxic forms or chelation of the metal ions required for their activation. These can be grouped into 2 categories -enzymatic and non-enzymatic [8, 15].

- **Enzymatic antioxidants** – Catalase (CAT), Superoxide dismutase (SOD), Glutathione peroxidase (GTPx), Thioreductase (TRX), Peroxiredoxin (PRX) and Glutathione transferase (GST). Enzymatic antioxidants act on specific substrates and are responsible for converting ROS and reactive nitrogen species to harmless species.

• **Non-enzymatic antioxidants** (small molecules) – Vitamins A, C and E, β-carotene and Glutathione. These non enzymatic antioxidants, act as oxidants and free radical scavengers.

Cells restore redox homeostasis by either activation or silencing of genes coding for defence enzymes, structural proteins and transcription factors. In addition to endogenous ones, exogenous antioxidants ingested through diet also play a role in maintaining the redox homeostasis. These act as chemopreventive agents by scavenging the ROS and enhancing host antioxidant defence systems against genotoxic carcinogens [65]. Antioxidants can protect DNA from oxidative damage, as indicated by a reduction in genotoxicity biomarkers such as micronuclei and chromosomal aberrations. The use of antioxidants from various natural and dietary sources, as antigenotoxic agents have been studied extensively.

2.5.2 Evidence of some natural products with genotoxic and antigenotoxic effects

Natural compounds can exhibit multiple biological activities such as antioxidant ability, metal ion chelation, modulation of DNA repair systems [128], inhibition of bioactivating enzymes and induction of detoxifying enzymes [3] and inhibition of cytochrome P 450 mediated activation of toxic compounds[7].

Ascorbic acid prevents oxidative DNA damage and Mn formation induced by heavy metals and radiation [129]. Flavonoids like quercetin, rutin and ursolic acid suppress the superoxide ion toxicity by scavenging it and enhance the production of DNA repair enzyme beta DNA polymerase, thereby increasing the cell survival [130]. Mozdarani & Kamali in 1998 [7] proposed that ROS scavenging ability is one of the mechanisms for the antigenotoxic activity of cimetidine against benzene.

Kilani-Jaziri *et al*., [131], reported the anti-microbial, antioxidant and antigenotoxic potential of *Cyperus rotundus*, could be due to compounds such as flavonoids and phenols. The antigenotoxic ability of Acai fruit pulp against doxorubicin was evidenced in studies conducted by Ribeiro *et al*., [132]. Flavonoids such as quercitin, rutin and terpenoids like ursolic acid have been reported to show antigenotoxic activities in different *in vitro* models [19]. Grape fruit juice is capable of reducing the concentration of blood cholesterol and also has efficient anticlastogenic properties [64]. *Myrciaria dubia* juice has antigenotoxic and antioxidant effects on mice blood cells [28]. Celiklar *et al*., in 2008 showed the antimutagenic activity of the green alga *Ulva rigida*[133]. Camel milk has shown anticlastogenic effects against the cisplatin induced micronucleation and anticytotoxic effects by restoring the mitotic index of bone marrow cells [134].

Hydroalcoholic extracts of Brassica leaves significantly reduced DNA damage inflicted by doxorubicin in mice [135]. Palmarosa and citronella essential oils [105] and Eicosapentanoic acid [65] show potential as adjuvants in chemotherapeutic regimen by virtue of their antigenotoxic, antioxidant and cytoprotective effects.

2.6 Review on Importance of Plant Products and Active Metabolites in Disease Therapy

Plants produce numerous secondary metabolites in response to environmental stimuli. These include phenols, carotenoids, alkaloids, amines, betalains, vitamins, terpenoids and other endogenous compounds. These compounds are also called phytochemicals. They protect the plants from diseases and grazing animals, as well as responsible for the colour and fragrance. Phytochemicals are stored in different parts of the plants. Epidemiological studies have revealed that many of these exhibit biological properties such as antioxidant, anti-inflammatory,

antitumour, antimutagenic, antimicrobial and antiviral activities. They can modulate enzymes involved in detoxification and hormone metabolism and stimulate the immune system [28, 106, 133, 136]. *Zuccagnia punctata* [26], *Cyperus rotundus* [131], Acai (*Euterpe oleracea*) [132], *Dendrobium* species[106], *Ocimum gratissimum* [137], and several other plant extracts are bestowed with potential antihelminthic, antibacterial, antifungal, and antiviral activities, as well as immunomodulation and cancer chemoprevention [21]. Vanillin [138], Ginseng [139], *Salvadora persica*[27] have been reported to exhibit multifunctional effects, including antimutagenic, antiangiogenic, anticolitis, and antianalgesic effects. Some natural substances are used in cosmetic formulations due to prominent characters like UV absorption ability [81].

Inhibitors of mutagenesis are also inhibitors of carcinogenesis. The incorporation of antimutagens through everyday diet, either in the form of fruits and vegetables or supplements, will be a very effective stratagem for prevention of cancer and genetic diseases [140].

2.7 General Therapeutic Importance of Plant Phenols and Saponins

2.7.1 Phenols

Phenolics are secondary metabolites found practically in all higher plants. They are a large group comprising of thousands of individual entities. Phenolic compounds in plants serve in diverse purposes like growth, pigments, defence against pathogens and environmental stresses and the list is still growing. They are of major importance concerned with human nutrition, ethnopharmacology as well as toxicology. Phenols are very good antioxidants and quench reactive radical intermediates in an array of molecular processes. They are considered as very good candidates for reverse pharmacology, a concept of studying activities of compounds from the state of proven safety and efficacy towards the molecular mechanism of their action [141].

2.7.1.1 Structure and Physicochemical properties

Phenolics, also termed as polyphenols consist of an aromatic ring with one or more hydroxyl groups. They exhibit great structural diversity ranging from monomers to polymer. They are formed from pentose phosphate and shikimate pathways. The number of hydroxyl ions the aromatic rings determines the antioxidant capacity [142]. They can be present as mono- and poly-saccharide conjugates or as esters and methyl esters. Their structure influences their absorption and metabolism, which in turn determines their biological properties. The phenolic compounds are further grouped into phenolic acids, quinones, tannins, stilbenes, flavonoids, lignans, coumarins and others.

2.7.1.2 Biological activities

Polyphenols have shown an array of important biological activities like antimicrobial activity, reduction of triglyceride deposition, protection against diabetes, cancer, cardiovascular inflammatory and allergic diseases [24, 25], majorly ascribed to their antioxidant capacity. Topical studies substantiate that the even very low concentrations of phenols or their metabolic products can wield modulatory effects on protein and lipid kinase cascades, crucial for cell cycle regulation [24, 142].

Phenolic acids and tannins are reported to have anticarcinogenic, antigenotoxic effects, as they protect DNA against free radicals, by blocking the sites of DNA vulnerable to electrophilic attack, inhibit enzymatic activation of pro-carcinogens and enhance enzymatic detoxification of xenobiotics [19, 23,24, 27, 133, 142]. They also influence the DNA repair pathway by modulation of gene expression and/or mRNA stabilization and inhibit DNA methylation and the formation of adducts. Phenolic substances may also protect biological systems through the reduction of absorption of toxic compounds due to cytochromeP450 inhibition [116, 143] and increasing the expression of the enzyme beta DNA polymerase needed for the error-free DNA repair [141].

Their possible role in cancer therapy is indicated by studies that revealed their capacity to elicit apoptosis in leukaemia cells [24, 142]. A study of a large number of Chinese medicinal plants with antitumour properties indicated that these herbs are very rich in content and variety of phenolic antioxidant components [23].

2.7.2 Saponins

Saponins are glycosidic compounds present in plants and possess a steroidal or triterpenoid aglycone and one or more carbohydrate moieties in their structure. The structural diversity of this group of compounds is responsible for their diverse properties. Plant saponins are extensively used as surface active and foaming agents in food and industrial purposes. They are known to be the active constituents of many plant based medicines. Legumes such as Soya, Green gram, Red gram, Rajma are rich in dietary saponins. Saponins used in health and industrial applications are obtained from Soap nut tree, Fenugreek, Alfalfa, Horse chestnut, Licorice, and Ginseng [30], [144–146].

2.7.2.1 Structure and physicochemical properties

Saponins are a diverse collection of plant secondary metabolites spread across more than 100 families. They are found in some marine organisms also. They possess a characteristic structure which is responsible for their amphiphilic nature. They are usually composed of two major parts, one of which is a liposoluble aglycone (also called sapogenin) and the other being one or more water soluble sugar moieties. The oligosaccharides are linked to the aglycone by a glycosidic linkage. The aglycones mostly consist of unsaturated C–C bonds. The lyobipolar nature of their constituents explains their ability to interact with cellular membranes and their soap-like behaviour in aqueous solutions, due to which they are used as surface active agents, hair cleansers detergents, wetting agents and emulsifiers. Depending on the type of aglycone skeleton, saponins are classified into triterpenoid

saponins and steroidal saponins. Steroidal saponins are found in monocotyledonous plants and triterpenoid saponins, in dicotyledonous flowering plants. The number of oligosaccharide chains classifies them as mono, di-, or tridesmosidic. The common sugar moieties in saponins are pentoses, hexoses or uronic acids. Saponins, due to their diverse and complex structures, demonstrate a broad spectrum of physico-chemical and biological properties [30, 144].

2.7.2.2 Biological activity of saponins

Saponins display a wide array of activities, including adjuvant, antibacterial [147], antioxidant [148],cytotoxic[149], antiparasitic, chemopreventive, diuretic, antiallergic, antigenotoxic, antihepatotoxic, anti-inflammatory, antimicrobial, antimutagenic and hypocholesterolemic properties[30, 145]. Extracts of plants containing saponins have been shown to be effective in the prevention and treatment of inflammation, infection, alcoholism, pre- and post-menopausal symptoms, cardiovascular[150] and cancer [144, 146, 151]. Sapogenins present in Agave are employed as raw materials in production of steroid drugs. They have immune stimulatory properties and function as adjuvants in vaccine formulations. Some of them isolated from Mexican yam are precursors for progesterone and oral contraceptives [152].

2.8 Literature review on Apocynin and Diosgenin

2.8.1 Apocynin

2.8.1.1 Properties of apocynin

Apocynin was isolated first from the roots of *Apocynum cannabinum* (Canadian hemp) in 1883 and later in 1971, from *Picrorhiza kurroa*, a perennial plant growing in the Himalayan mountain ranges in India, Nepal, Tibet, and Pakistan [9].Extracts from this plant are used widely in the herbal medicines in India, Sri Lanka, Nepal, China and other countries for treating diseases of heart, liver, joints, asthma and other lung problems [153].

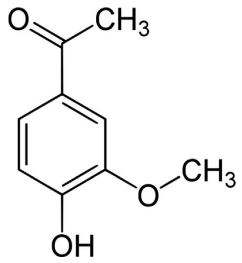


Figure 2.8.1 Chemical structure of apocynin

Its IUPAC name is 1-(4-hydroxy-3-methoxyphenyl) ethanone and also known by the synonyms 4-hydroxy-3-methoxyacetophenone and acetovanillone (Figure 2.8.1adapted from Wong *et al.*[154]). It is a methoxy-substituted catechol with MW 166.17 and melting point 115%C. It has a mild odour similar to vanilla [9]. It is a proven inhibitor of NAD(P)H oxidases and concomitant ROS production in several experimental systems [153, 155].

Apocynin is a prodrug, which was found to undergo dimerization into diapocynin on metabolically reacting with H_2O_2; myeloperoxidase acts as catalyst in formation of this dimer [156]. Another remarkable feature of this phenolic substance is that it has very low toxicity in experimental animals (LD50 value of 9g/kg) [157] .

2.8.1.2 Pharmacological effects of apocynin in various experimental models

P.scrophulariiflora and *P. kurrooa* are widely used in different ethnic medicinal systems in treatment of immune-related diseases [158]. Apocynin has been considered a miraculous molecule for the multitude of pharmacological effects it exhibits. This compound has shown promising effects in animal models of disease as well as cell culture studies where ROS are involved, including arthritis, septic shock, asthma, hypertension and other

cardiovascular diseases, airway inflammation and endotoxin induced lung injury [17,159,160]. The anti-inflammatory properties of apocynin against immune mediated disorders like rheumatoid arthritis are emphasized by inhibition of oxidative burst in neutrophils *in vivo* [161] and TNFα production in peripheral blood mononuclear cells in culture [162].

Its protective effects on various liver implications are well recognized, for example, in hepatic ischemia/reperfusion injury, hemorrhagic liver injury and hypercholesterolemia [76]. In certain experimental systems it extended antioxidative protection to the brain cells microglia and has shown promising results in alleviating the symptoms of neurodegenerative disorders, such as Multiple sclerosis, Parkinson's disease, ALS and Alzheimer's disease [156, 161].

2.8.1.3 Mechanism of action of apocynin

Apocynin is an established inhibitor of Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, NOX). NADPH oxidase is a multimeric enzyme responsible for conversion of oxygen molecules to superoxide anion [O^{2-}.] as well as capable of generation of H_20_2. Apocynin blocks the expression of cytosolic components p47phox and gp91phox of this enzyme and also blocks their translocation to the membrane fraction, there by obstructing assembly of this enzyme complex [159].

Some researchers state that apocynin does not always act as NADPH oxidase inhibitor [163] and exhibits other modes of action such as blocking the production of thromboxaneA2 in pulmonary macrophages, inhibition of cytochromeP450 activity, interferes with cytoskeletal arrangement in PMN granulocytes [164], inhibits activation of NF-κB and AP-1[160], which are redox regulating transcription factors and reduces cytokine expression in lymphocytes [165]. It increases expression of antioxidant defence

enzymes [76] and acts as a strong scavenger of radical oxygen species and reactive nitrogen species [154] and also non-radical oxidants like H_20_2 and HOCl [159].

Pro-oxidant nature of apocynin is suggested by results of some studies where it enhances NO synthesis, perturbing the redox balance [166] and caused manifold increase in oxidation of GSH and coenzyme NADPH [167].

2.8.2 Diosgenin

2.8.2.1 Properties of diosgenin

Diosgenin is a plant secondary metabolite and its chief sources are Fenugreek (*Trigonella foenum graecum*), Wild yam (*Dioscorea villosa*), *Solanum incaunm*, and *S. xanthocarpum* [168]. Diosgenin [(25R)-5- spirosten-3h-ol] or [(25R)-spirost-5-en-3b-ol] (Figure 2.8.2-adapted from Das and Bharali [169]) is a dioscin aglycone. It is a spirostanol or steroidal saponin with a hydrophilic sugar linked to the hydrophobic aglycone. It is structurally analogous to other steroids like cholesterol.

2.8.2.2 Pharmacological effects of diosgenin

Existing records prove that Fenugreek seeds and wild yam tubers are used to treat metabolic diseases like arthritis, cancer, diabetes, gastrointestinal disorders, inflammation and high cholesterol [34]. The tubers of *Diascorea* species are well known conventional medicines for diarrhoea, diabetes, skin problems, and rheumatism, in China and Zimbabwe. Records of Aztec and Mayan civilizations mention use of wild yam tubers as relieve pain. These tubers are commercial sources of secondary metabolites used in the pharmaceutical industry and general medicine; the most important ones are steroidal saponins, including diosgenin. Extracts of Diascorea tubers have shown major biological functions such as antitumour and anticancer properties, antimicrobial activity, hypoglycemia, anti-hypertension, antifatigue , antioxidant, and reno-

hepato-protection, according to clinical supplement trials and animal/ cell model system tests [170]. Fenugreek seeds were used to induce childbirth and as medicine for gynaecological inflammation. The anti-diabetic and hypocholesterolemic activity of Fenugreek are attributed to diosgenin [34,171].

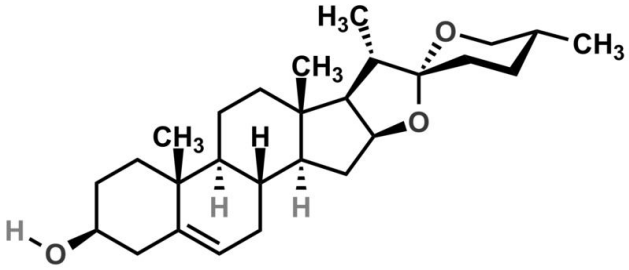


Figure 2.8.2 Chemical Structure of Diosgenin [(3β, 25R)-Spirost-5-en-3-ol]

Diosgenin exhibits anti-invasive and antiproliferative action against cancers of the breast, bone, colorectum, liver and also leukaemia mediated by a range of mechanisms [172]. It is used in oral contraceptive pills due to its oestrogenic effects on mammalian glands. It has been used in skin care formulations to reduce the signs of aging in relation with keratinocyte proliferation and has inhibitory effects against melanogenesis[173].It is widely in use as precursor for various synthetic steroidal harmones like progesterone, and steroidal anti-inflammatory agents like cortisone [174]. Diosgenin enhanced the apoptotic effects of chemotherapy drugs Paclitaxel and Doxorubicin [172]. It significantly increases the hepatic GST and other detoxifying enzymes [169]. Oral pretreatment with diosgenin reduces the micronucleation, chromosomal aberrations, DNA damage and lipid peroxidation in hamsters treated with 7, 12-DMBA [175]. It was suggested to act as a unique chemotherapeutic modulator in the prevention and treatment of HER-2 overexpressing cancer [176].

2.8.2.3 Mechanism of action of diosgenin

The anti-cancer effects of diosgenin was shown to be due its structure which includes a hetero-sugar and a double bond between the carbon atoms at position 5and 6 [34]. Diosgenin is said to target multiple stages like proliferation, angiogenesis, metastasis and immunosuppression in cancer cell cultures and *in vivo* tumour models. Some studies have indicated that it involves activation of p53, modulation of cell proliferation and apoptotic signalling, for example the STAT-3 pathway, and enhancement of certain cell death receptors [168]. It arrested the cell cycle in G2/M phase in HCC, Erythroleukaemia and Leukaemia cells but in Osteosarcoma and hepatoma cells, arrested the cell cycle in G1 phase [177]. These studies put forward that the mechanism of action of diosgenin may vary with the cell type. In HER-2 over expressing cancer cells diosgenin suppressed the expression of FAS and modulate Akt, mTOR and JNK phosphorylation [176].

2.9 Cell lines – HepG2 and CHO-K1

2.9.1 HepG2 cell line

HepG2 is an immortalised human liver cancer cell line derived from a teenaged Caucasian American male. These cells have a modal chromosome number of 55. They do not grow into tumours in nude mice. They secrete a collection of major plasma proteins, such as albumin, transferrin, fibrinogen and plasminogen. This cancer cell line is acknowledged for their phase I and II biotransformation enzyme activities which regulate the metabolism related to activation and/or detoxification of xenobiotics and carcinogenesis. The cells morphologically are similar to the cells of liver parenchyma from which they originate. Hence the HepG2 cell line is taken to be an efficient model for *in vitro* investigations of the xenobiotic metabolism and toxicity in the liver, very close to the *in vivo* situation [178]. HepG2 cells have been extensively used in studying the genotoxic/antigenotoxic and cytotoxic/cytoprotective effects of several natural and synthetic compounds using various assay systems [170,179].

2.9.2 CHO-K1cell line

CHO (Chinese Hamster Ovary) cells are a cell line derived from cells of the ovaries of Chinese hamsters (*Cricetulus griseus*). Due to their petite size and low chromosome number (2n=22) for a mammal, Chinese hamsters are popular as a convenient model for tissue culture and cytogenetics. The original CHO cell culture was derived from a Chinese hamster at the Boston Cancer Research Foundation by T. Puck. CHO cells are extensively used as experimental models for mammalian systems in medical and pharmaceutical research. Moreover, they are regularly used for commercial production of therapeutic recombinant proteins. Numerous lines with different characteristics have been developed from the first cell line. A frequently employed CHO derivative is CHO-K1; these cells have less DNA than the original CHO cells [180] making them more suitable for studies involving gene expression, toxicity assessment, and virology, as well as Prion disease [181,182,183].

CHAPTER - 3

3.1 Drugs and Chemicals

Various important drugs/chemicals used in the study are listed below,

- Apocynin (Natural Remedies, Pvt. Ltd Bangalore, Batch No.T12G190- 2014)
- Diosgenin (Natural Remedies, Pvt. Ltd Bangalore, Batch No.T12K015- 2013)
- 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) (Hi-Media Pvt. Ltd.)
- Acetoorcein (Hi-Media Pvt. Ltd.)
- Bovine Serum Albumin (SD Fine chemicals)
- Cisplatin (Cipla, India)
- Cyclophosphamide (Brand Name-Uniphos 500, United Biotech, India, Batch No.UPDJ3B7-2013)
- DMEM media (Hi-Media Pvt. Ltd.)
- Geimsa stain (Merck India Pvt. Ltd.)

- May-GRUNWALDs Stain(Lobo Chemicals, Mumbai, India)
- MTT (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) powder
- (Hi-Media Pvt. Ltd.)
- RNase A (Hi Media Pvt. Ltd)
- Thiobarbituric acid (Hi Media Pvt.Ltd.)
- Trichloroacetic acid(Hi Media Pvt., Ltd.)
- Trypsin (Hi Media Pvt. Ltd.)
- DPX mountant and Cedar oil (SD Fine Chemical Ltd., Mumbai, India)
- Agarose (Hi Media Laboratories Pvt.Ltd., India).

Other chemicals of lab reagent grade as per requirement were purchased from commercial suppliers and used (Methanol, Acetic acid, Potassium di-hydrogen phosphate, Sodium chloride, Sodium di-hydrogen phosphate, Di-sodium hydrogen phosphate, EDTA, DMSO, NaOH, Hydrochloric acid).

cont...

3.2 Equipment

Various instruments used in the study are listed in the Table 3.2.1.

Table 3.2.1
List of important equipment used in the present study

Instrument	Make
Centrifuge	Hettich Rotina 420R
Camera microscope	DeWinter 400x
Deep Freeze (-80°C)	Revco
Deep Freeze (-20°C)	Ariston
Deionized Water Device	Barnstead
Distilled Water Device	MES ultrapure
Electrophoresis	Biometra Analitik
Electrophoresis Power Supply	Power Pack P 25
Fluorescent Microscope	Leica
Gel-Doc chamber	Bio-rad
CO_2 Incubator	Heraeus Instruments
Inverted Microscope	Leica
Laminar Air Flow	Heraeus
Magnetic Mixer	Stuart Scientific
Micro Centrifuge	Heraeus
Micropipettes 1-10 µl, 0 5-40 µl, 40-200 µl, 200-1000 µl, 1-5ml	Finnpipette,Gilson, Biohit
Microfilter	Millipore
Neubauer Slide	Marienfeld
PH Meter Cyberscan Scale	Schimadzu Libror
Slides (26x76mm)	Marienfeld
Spectrophotometer	Schimadzu Libror
Micro Plate reader	Tecan
Ultrasonic Bath	Transsonic 460/H
Vortex	Heidolph 2000
Water Bath	Termal® Laboratory Tools

3.3 In vivo Mice Bone Marrow Micronucleus Assay

3.3.1 Experimental animals

Swiss albino mice were chosen for the *in vivo* experimental studies. Healthy mice of either sex, which are of eight weeks age,

(weight 25±5 g) were used. Animals were maintained at a temperature of 25±2°C, 12 h of natural light in standard lab conditions. Drinking water and pellet diet (Lipton India) were provided *ad libitum*. *In vivo* experiments were performed with permission from the animal ethical committee of the institute (Certificate Ref.No.SSCPT/IAEC.Clear/150/2013-14).

3.3.2 Experimental design

Evaluation of apocynin pretreatment in cyclophosphamide induced genotoxicity

The experimental protocol suggested by Hayashi *et al.* [184,185] with some modifications was adopted. Five groups of six mice each were formed and subjected to various treatments as listed below-

Group 1: Normal/vehicle control --received saline intraperitonially for 5days 10ml/Kg body weight.

Group 2: Plant active constituent control--received apocynin 200µg/kg body weight for 5days i.p.

Group 3: Clastogenic control (positive control) —given CP alone 50mg/kg body weight i.p. in a single dose.

Group 4: Treated with apocynin 100µg/kg body weight for 5 days and 1hr after the last dose injected intraperitonially with a single dose of CP 50mg/kg body weight.

Group 5: Treated with apocynin 200µg/kg body weight for 5 days and 1hr after the last dose injected intraperitonially with a single dose of CP 50mg/kg body weight.

Evaluation of diosgenin pretreatment in cyclophosphamide induced genotoxicity

Group 1: Normal/vehicle control --received saline intraperitonially for 5days 10ml/Kg body weight.

Group 2: Plant active constituent control --received Diosgenin 200µg/kg body weight for 5days i.p.

Group 3: Clastogenic control--received CP alone 50mg/kg body weight i.p. in a single dose.

Group 4: Treated with diosgenin 100µg/kg body weight for 5 days and 1 hr after the last dose, injected intraperitonially with a single dose of CP 50 mg/kg body weight.

Group 5: Treated with diosgenin 200µg/kg body weight for 5 days and 1 hr after the last dose, injected intraperitonially with a single dose of CP 50 mg/kg body weight.

3.3.3 Experimental procedure

Collection of samples:

The animals were sacrificed after 24hrs of CP administration by cervical dislocation under light anaesthesia. Before sacrifice the animal's blood samples were collected for the estimation of WBCs, RBCs and haemoglobin content. The liver samples were collected for the estimation of LPO and GSH. The femur and tibia were also excised (Figure 3.3.1 and Figure 3.3.2)

Reagents:

- Phosphate buffered saline –

Solution A- Di-sodium hydrogen phosphate – 2.136g in 100ml of distilled H_2O; solution B- Sodium di-hydrogen phosphate – 2.344g in 100ml; solution C- Saline – 0.9% sodium chloride 41ml of Sol.A + 9ml of Sol.B + 50ml of Sol.C are mixed sequentially and then pH is adjusted to 7.2.

- 5% bovine serum albumin- BSA 0.5g in 10ml of PBS.
- May-GRUNWALD stain: 0.02% solution in methanol.
- Phosphate Buffer (pH 6.8)

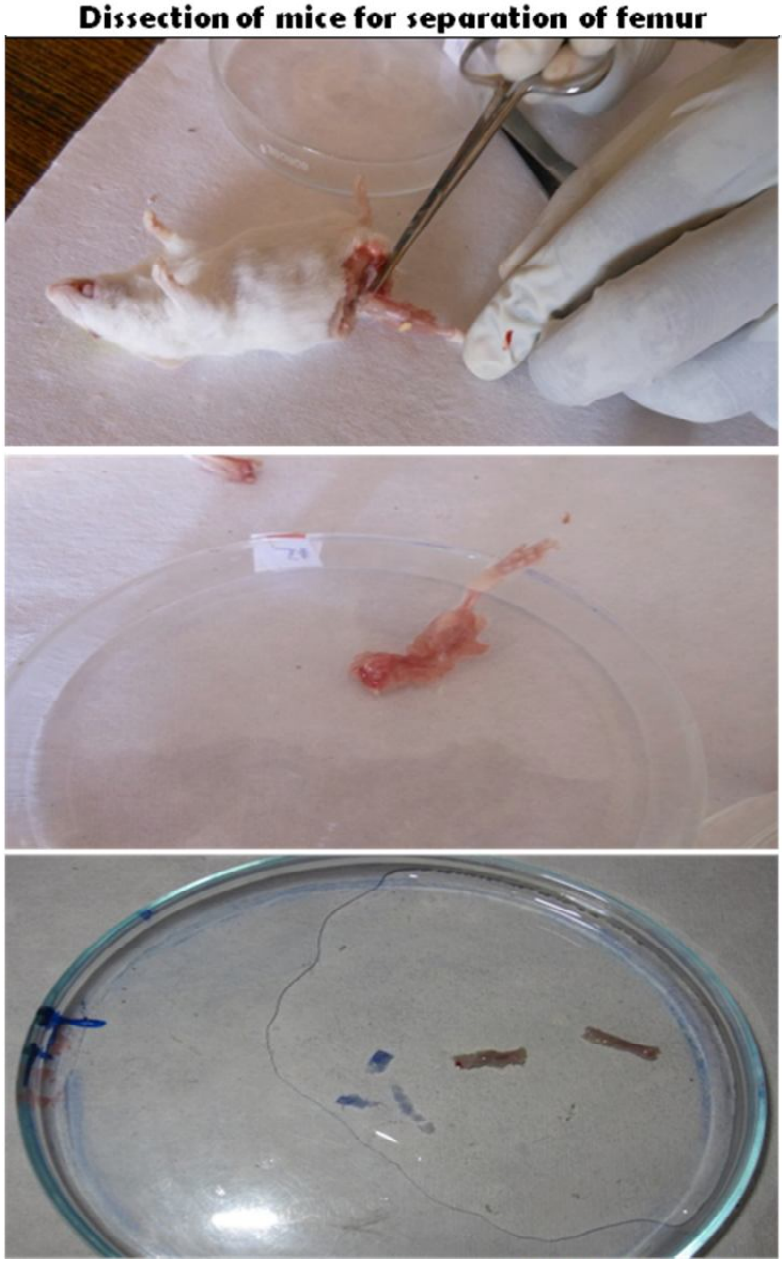


Figure 3.3.1 Dissection of mice for separation of femur

Figure 3.3.2 Collection of liver tissue

Bone marrow extraction:

1. After sacrifice of the animals, both the intact femurs were removed *in toto*, by cutting through pelvis and tibia.
2. The muscular tissue attached to the femurs was cleared by rubbing with gauze.
3. The distal epiphysial region and rest of tibia were removed by gentle traction.
4. Both ends of the femur were trimmed till a small opening to the marrow canal was seen.
5. A disposable plastic syringe with the needle of appropriate size was used to carefully flush through the bone with 5% BSA into a centrifuge tube till almost all the marrow was collected, as a fine suspension.

Preparation of smear:

1. The bone marrow cell suspension was centrifuged at 1000rpm for 5min.
2. The supernatant was decanted.
3. Cells in the pellet were carefully suspended in a few drops of serum by aspiration with a siliconized pasteur pipette.
4. A small drop of this viscous suspension was placed at the end of a clean glass slide and made into a smear using another glass slide held in a slanting position. The smear was allowed to air dry.

Staining and counting of the cells:

1. Air dried slides were first stained with May-Grunwald stain diluted with phosphate buffer pH 6.8(1:1) for 12-15min.
2. Then the slides were transferred to Giemsa stain diluted with phosphate buffer (1:6) and stained for 12min.
3. Phosphate buffer was used to rinse the stained slides 2-3min and blotted with filter paper.
4. Back side of stained slides was cleaned with methanol and fixed permanently with the help of DPX mountant.

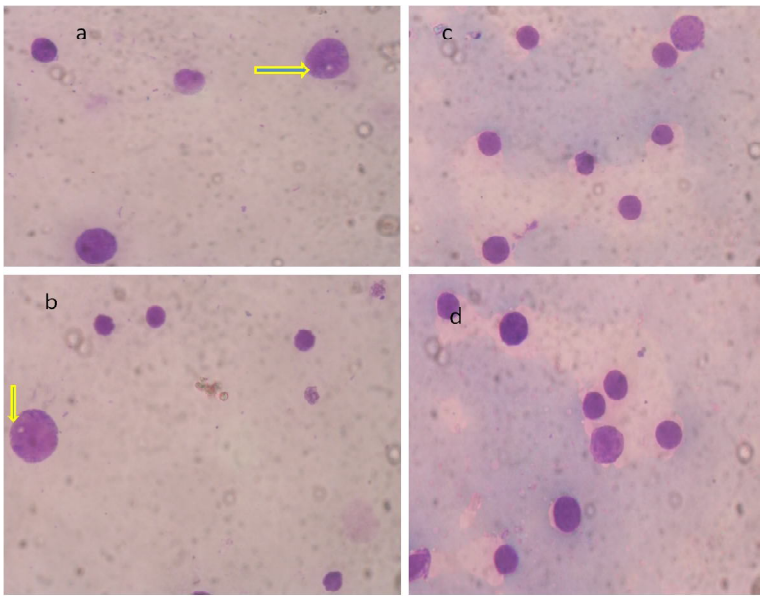


Figure 3.3.3 Presence of micronuclei in PCEs

a and b – Micronuclei, indicated by arrows, seen in PCEs, observed in mice treated with CP alone; c and d – PCEs without appearance of micronuclei

5. Stained slides were observed under 100X oil immersion lens. About 1000 polychromatic erythrocytes (PCE) were scored for the presence of micronuclei (Mn) per animal in each group (Figure 3.3.3).

3.4 Measurement of haematological parameters

3.4.1 Determination of haemoglobin (HB) in blood

Principle: Haemoglobin content was measured using haemoglobinometer. Anticoagulated blood is mixed with 0.1N HCl to form acid haematin. The acid haematin so formed is diluted with distilled water until the colour matches with the standard present in the calibration tube. The final reading in terms of percentage HB is directly obtained from the markings on the calibration tube [186].

Procedure:

1. 0.1N HCl was taken in the graduated tube up to the mark 20.
2. 20mm^3 of anticoagulated blood was drawn into haemoglobin pipette and transferred in to graduated tube containing HCl and mixed well.
3. The mixture was allowed to stand for 10 min. After 10 min the solution was diluted by adding drop by drop distilled water with constant stirring until the colour matched with the standard.
4. The percentage haemoglobin was read from the scale on the graduated tube.

3.4.2 Determination of total red blood cell (RBC) count

Principle: Whole blood is diluted with an isotonic RBC diluting fluid to avoid lysis of red cells. Then number of RBC in a known volume and known dilution of the blood sample is counted using a haemocytometer or Nuebauer counting chamber.

Procedure:

1. Using RBC pipette, blood was drawn up to 0.5 graduations. Any excess blood from the tip was wiped off.
2. The pipette was then filled up to the mark 101 with RBC diluting fluid and mixed for 1 min thoroughly.
3. The first few drops were discarded and then the fluid was charged onto the counting chamber of haemocytometer.
4. Total number of RBC in all the four corner squares and one central square was counted using 45 X objectives. The average number of cells/mm^3 was calculated using the formula

$$Total\ RBC\ count = Total\ No.of\ cells\ in\ the\ RBC\ counting\ area \times 1000\ cells/mm^3$$

(1000 is the volume of RBC counting area)

3.4.3 Determination of total white blood cell (WBC) count

Principle: Whole blood is diluted with a diluting fluid which haemolyses RBC but leaving all the nucleated cells intact. The number of WBC in a known volume and known dilution of the blood sample is counted using a Nuebauer counting chamber.

Procedure:

1. Using WBC pipette, blood was drawn up to 0.5 graduations. Any excess blood from the tip was wiped off.
2. The blood is diluted with WBC diluting fluid up to the mark 11 and mixed thoroughly for one minute.
3. The first few drops were discarded and the fluid was allowed to flow on to the counting chamber of haemocytometer.
4. Total number of cells in all the four corner squares of WBC counting area was counted using 10X objectives. Total number of WBC was calculated using the formula

$$Total\ WBC\ count = Total\ No.of\ cells\ in\ the\ WBC\ counting\ area \times 50\ cells/mm^3$$

(50 is the volume of WBC counting area).

3.5 Measurement of Oxidative stress parameters

3.5.1 Measurement of LPO concentration

Principle: Lipid peroxidation is a process of oxidative degradation of polyunsaturated lipids containing a C=C bonds. Malondialdehyde (MDA) or Thiobarbituric acid reactive substance (TBARS), formed from the deterioration of fatty acids, is a well established index for measuring the level of peroxidation. It undergoes chromogenic reaction with thiobarbituric acid to give a pink coloured end product which can be quantified at 532 nm. [77].

Reagents:

TBA-TCA-HCL reagent: TCA 15%w/v, TBA 0.375 %w/v in 0.25N HCl. This solution needs mild heating for dissolving the TBA.

Procedure:

1. The liver was homogenized and the homogenate, incubated with TBA-TCA-HCL reagent for 15 min at 95 ºC.
2. Then the mixture was allowed for cooling, centrifuged to remove the precipitates and the supernatant collected, absorbance of which was measured at 532 nm against suitable blank.
3. The amount of MDA was calculated using the extinction coefficient ε = 1.56 x 105 M-1 cm-1 and expressed as nmol/g of wet tissue weight.

3.5.2 Determination of Hepatic GSH contents

Principle: This is a colorimetric assay based on reaction the of acid soluble sulphydryl groups of GSH and other thiols, with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) forming an yellow coloured complex. The coloured complex is quantified by measuring the absorbance at 412 nm [187] .

Reagents:

- Trichloroacetic acid (TCA) 10%
- 0.2M Sodium Phosphate buffer(PB) pH 8.0 (0.218g NaH_2PO_4 and 2.641 g Na_2HPO_4 in 100 ml distilled H_2O)
- 0.6mM DTNB -pH 8 (11.9mg dissolved in 50ml PB)

Procedure:

1. Liver tissue was homogenized and 0.5ml of homogenate was mixed with 0.1ml of 10% TCA and allowed to stand on ice for a few minutes.
2. The mixture was centrifuged at 3000g for few minutes to pellet the precipitate.
3. The supernatant was collected and 0.3ml was mixed with 0.7ml of 0.2M phosphate buffer and 2ml DTNB solution.
4. After 10 min of incubation, the absorbance at 412 nm was measured using a blank, which contained all the reagents except for the tissue homogenate.

***3.6 Allium cepa* root tip meristem assay**— for the evaluation of effects of apocynin on cyclophosphamide induced genotoxicity

Onion bulbs were purchased and sundried for a week. Healthy bulbs without fungal attack of approximately 20g were chosen, the dried outer scales were removed and the dried roots were cut retaining the root primordia. The bulbs were placed in disposable plastic cups immersing the root primordia in plain drinking water for three days allowing roots to emerge, at room temperature (around 28°C). Water was changed periodically every 24hrs and when the rootlets reached a length of 2-3cm, they were exposed to different treatments mentioned in Table 3.6.1. [88,188,189].

Experimental design:

Table 3.6.1
Experimental design for *Allium cepa* root meristem assay

S.No.	Treatments (n=3)
1	Negative control (plain drinking water)
2	Positive control (cyclophosphamide 2mg/ml in water)
3	CP 2mg/ml +apocynin 100µg/ml in water
4	CP 2mg/ml +apocynin 200µg/ml in water
5	Apocynin 100µg/ml in water
6	Apocynin 200µg/ml in water

Reagents:

- Carnoy's fixative (3:1 Ethyl alcohol: Acetic acid)
- Acetoorcein
- 1N HCl

Experimental procedure:

1. After 24hrs of exposure to the treatments, the roots were rinsed with plain water and root tips of about 1cm length were collected.
2. The root tips were fixed in Carnoy's fixative for 24hrs and thereafter preserved in 70% alcohol.

3. The preserved root tips were then subjected to microscopic cytogenetic analysis. Root tips of about 2mm length were hydrolysed in 1N HCl and then stained with Acetoorcein.
4. Stained tips were squashed on glass slides under cover slips to spread the cells.
5. Dividing cells in all stages of mitosis i.e., prophase, metaphase, anaphase and telophase were counted under DeWinter camera microscope at 400X magnification.
6. Two root tips showing at least 10 dividing cells per 1000cells were selected from each bulb (replication) for analysis. 1000 cells per root tip were screened totaling to 6000 cells per treatment.
7. The mitotic index was calculated as follows-

$$MI = \frac{n_d}{n_t} \times 100\%$$

n_d—number of dividing cells and n_t—total number of cells

8. The number of cells showing mitotic abnormalities like lagging chromosomes, spindle abnormalities, adherent nucleus, anaphasic bridges and broken chromosomes were also counted per 1000 cells in each root tip.
9. The frequency of chromosomal aberrations was calculated as follows—

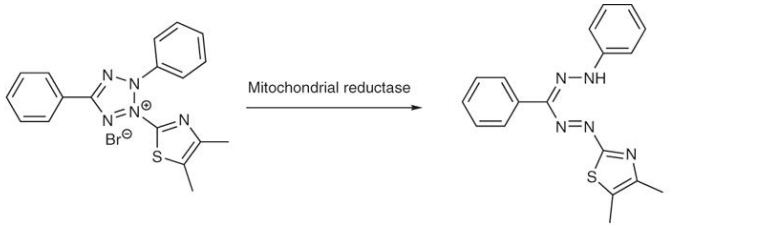
$$fCA = \frac{n_a}{n_t} \times 100\%$$

n_a is the number of aberrant cells

3.7 MTT Assay —for assessing the cytotoxic /anticytotoxic properties of apocynin and diosgenin

Principle: The key component MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is a tetrazolium salt which dissolves in media or salt solutions forming a yellowish solution, in the absence of phenol red. Mitochondrial

dehydrogenases in living cells cleave the tetrazolium ring in dissolved MTT and form insoluble purple formazan (Figure 3.7.1). This water insoluble formazan precipitate in the cells and the culture medium can be solubilised with DMSO or other solvents like acidified isopropanol or ethanol. The colour intensity of the resulting solution is spectrophotometrically measured and is directly proportional to the number of viable cells in the culture, indicating the extent of cytotoxicity of the test compound [96,190].



(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide))

MTT

(2E,4Z)-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan

(Formazan)

Figure 3.7.1 Chromogenic conversion of MTT to Formazan

Reagents:

- MTT solution - 5mg /10ml of 1XPBS (the solution should be filtered using a 0.2µ filter and can be stored at 2-8°C)
- DMSO

Procedure:

1. DMEM media with FBS was used to culture the selected cell lines till they reach about 70-80% confluence. The cells were then harvested and checked for viability.
2. The harvested cells were then pelleted by centrifugation.
3. The pellet was resuspended in DMEM to adjust to the desired cell number.
4. About 10,000 cells / well were seeded in a 96 well plate and incubated in CO_2 incubator for 24 hrs at 37°C, 5 % CO_2 concentration.
5. Then the samples (apocynin/diosgenin/cisplatin) to be tested

were added from 0 – 120 µM/ml concentration in DMEM without FBS & are incubated for 24 hr for calculation of IC50 values.

6. In case of combination/simultaneous treatment, the test substances were added asmentionedin Table 3.7.1 and Table 3.7.2.

Table 3.7.1 Design of experiments for combination treatments with apocynin and cisplatin

S.No.	Treatment
1	Control
2	Apocynin (30µm)+ Cisplatin(30µm) - 1:1
3	Apocynin (30µm)+ Cis(60µm) - 1:2
4	Apocynin (60µm)+ Cis(30µm) - 2:1

7. After incubation with test samples, 100 l/well (50 µg/well) of the MTT solution was added and incubated for 3-4 hours.
8. The MTT reagent was discarded by removing with a pipette gently, without disturbing the cells.
9. 100 µl of DMSO was added to each well to rapidly the formazan
10. Absorbance at 590 nm was obtained using a plate reader.
11. The cell inhibition was calculated as follows

$$\%\ Inhibition = 100 - \left(\frac{OD\ of\ Sample}{OD\ of\ Control}\right) \times 100$$

Table 3.7.2 Design of experiments for combination treatments with diosgenin and cisplatin

S.No.	Treatment
1	Control
2	Diosgenin 25µM + Cisplatin 30µM
3	Diosgenin 50µM + Cisplatin 30µM
4	Diosgenin 100µM + Cisplatin 30µM

3.8 Haemolysis assay — for understanding the haemocompatibilty of apocynin

The assay was performed according to the international standard ISO 10993-4:2002 [191].

Principle: An erythrocyte suspension and the test substance are co-incubated at a defined pH, at 37^{0}C to allow for any red blood cell disruption. Intact cells are pelleted out and haemoglobin content of the supernatant is read at 540nm. The percent haemolysis is then quantified relative to positive control samples lysed with a detergent like 1% TritonX or 1% SDS.

Procedure:

Isolation of erythrocytes:

1. 5ml of blood was anti-coagulated with 5.4mg of EDTA and subjected to centrifugation at 1000rpm, 10min, 4^{0}C.
2. The overlying Plasma and the white buffy layer were carefully aspirated, using a pipette.
3. The RBC in the pellet were washed thrice with PBS, stored at 4^{o}C and used ore 6h.

Protocol for estimating the haemolysis:

1. 50µl of Erythrocytes suspension diluted with PBS was taken into 2ml fresh eppendorf tube and 100µl of test samples as listed in the Table 3.8.1 were added.
2. The tubes were incubated for 60 min in water bath at 37^{0}C.
3. 850 µl of 1XPBS was added to make up the volume to 1 ml.
4. The reaction mixture was subjected to centrifugation at 300 rpm for 3 min.
5. Absorbance of the supernatant containing released haemoglobin was measured at 540 nm in a spectrophotometer.

Table 3.8.1 Experimental design for haemolysis assay with apocynin and cisplatin

S.No.	Treatment
1	Positive control 1% SDS
2	Negative control 1X PBS
3	Cisplatin 30µM
4	Cisplatin 30µM + apocynin 30µM
5	Cisplatin 30µM + apocynin 60µM

3.9 DNA fragmentation assay to test the protective ability of apocynin against oxidative DNA damage

Principle: Nuclear DNA in dying cells is cleaved by endonucleases into nucleosomal units, of about 180bp length or multiples of it. When such DNA is electrophoresed on an agarose gel, these fragments form a DNA ladder.

Reagents:

- Lysis buffer (pH 7.8) [Tris-HCl 10mM, pH8; EDTA 20mM, pH8.0; 0.2% TritonX-100; 4M NaCl]
- TE buffer (EDTA 1mM, pH 8.0; Tris-HCl 10mM, pH 7.4)
- Trypsin
- RNase A
- 3M Sodiun Acetate
- 100% Ethanol
- Phenol-Chloroform-Isoamylalcohol

Protocol:

1. Selected cells (CHO-K1) were inoculated at a concentration of 10^6 per 35 mm dish and allowed for incubation in CO_2 incubator for 24 h at 37°C and 5% CO_2.

Table 3.9.1 Experimental design for DNA fragmentation assay with apocynin and cisplatin

Lane No.	Treatments
1	DNA ladder
2	Cisplatin alone
3	Cisplatin 30µM
4	Apocynin 30µM + cisplatin 30µM
5	Apocynin 60µM + cisplatin 30µM

1. The confluent cells grown after 24 hrs of incubation were treated with the treatments as in Table 3.9.1.
2. After treatment, cells were collected by trypsinization and centrifugation at 2000 rpm for 5 min to collect adherent as well as floating cells.
3. The pellet was resuspended in 0.5ml lysis buffer (pH7.8), vortexed vigorously and incubated for 5min at 50°C.
4. To the lysate, 0.5ml of Phenol-Chloroform-Isoamylalcohol mixture was added and mixed for 2-3 minutes. Then centrifugation at 10000rpm for 15min at 4°C was done.
5. The upper aqueous layer was taken in a new tube, to which double the volume ice cold 100% ethanol and also 3M sodium acetate was added so as to get a final concentration of Sodium acetate 0.3M. It was incubated for 5-10min. at room temperature.
6. The tubes were centrifuged at 10000rpm for 15 min.
7. After removing the supernatant, the pellet containing the DNA was washed in 70% Ethanol by centrifugation at 5000 rpm for 10 minutes.
8. Supernatant was removed and the DNA pellet was air dried and was finally dissolved using TE buffer, and subjected to electrophoresis on a 2% agarose gel at 100V for 50min.
9. The gel after electrophoresis was then visualized in a Gel documentation chamber and photographed.

Statistical Analysis: All the data were expressed as mean±SEM for each group and statistically analysed by one-way ANOVA followed by Tukey's post-test, using Graph pad prism-7 statistical package. P values of d" 0.05 were considered to be statistically significant.

CHAPTER - 4

4.1 Genotoxic and Antigenotoxic Effects of Apocynin

4.1.1 Influence of apocynin on induction of MnPCEs in mice bone marrow

The results of micronucleus assay are expressed in Table 4.1.1.and Figure 4.1.1 Administration of cyclophosphamide in the dose of 50mg/kg body weight i.p induced a significant increase in the number of micronucleated polychromatic erythrocytes (MnPCEs) as compared to the bone marrow cells of the mice in the normal control group. Pre-treatment of apocynin at concentrations of 100 and 200 µg/kg, bodyweight (p.o.) to a cyclophosphamide administered mice showed a considerable decrease in micronuclei formation when compared to cyclophosphamide alone treated mice. Pre-treatment with apocynin alone in the dose of 200µg/kg bodyweight (p.o.) of experimental mice did not induce micronucleation in the bone marrow cells of the mice.

4.1.2 Effect of apocynin on haematological parameters in experimental animals

4.1.2.1 Total WBC count

CP significantly lowered the total WBC count considerably as compared to the normal control. Apocynin alone does not have any significant lowering effect on the number of WBC as compared to normal control. But in animals treated with apocynin along with CP, the WBC count was significantly restored when compared to animals treated with CP alone (Table 4.1.1; Fig. 4.1.2).

4.1.2.2 Total Erythrocyte count and Haemoglobin percentage

The total number of RBC and %HB content in the various experimental groups are not significantly altered by any of the treatments than that of the normal control (Table 4.1.1; Fig. 4.1.3 and Fig. 4.1.4).

4.1.3 Effect of apocynin on oxidative stress parameters

The results of studies conducted to analyse two of the oxidative stress parameters namely lipid peroxidation (LPO) and level of reduced glutathione (GSH) are presented in Table No.4.2.2

4.1.3.1 Effects of apocynin on the level of lipid peroxidation

The LPO in the hepatic tissues of experimental mice subjected to various treatments was assessed by quantifying the formation of MDA, a by-product of peroxidation of lipids. The concentration of MDA is directly proportional to lipid peroxidation. The lipid peroxidation levels in mice treated with cyclophosphamide alone were significantly higher when compared to normal control animals, with concomitant decrease in the GSH. Oral pre-treatment with Apocynin significantly prevented the cyclophosphamide induced lipid peroxidation and maintained the normal status in a dose dependent manner. Apocynin by itself did not cause enhancement of LPO. The influence of the treatments on LPO is graphically represented in Fig.4.1.5.

4.1.3.2 Estimation of the level of reduced glutathione

Another indicator of oxidative stress analysed was the concentration of reduced hepatic glutathione in the mice. Lower levels of GSH point towards higher levels of oxidative stress. Induction of oxidative stress in the hepatic tissues was evident in the mice subjected to CP treatment, by the significantly lower GSH content than in the mice of normal control group. While apocynin alone did not lead to decline in GSH, it could effectively replenish the GSH concentrations in CP treated animals, demonstrating lower levels of hepatic oxidative stress. This is an indication of apocynin being able to counter the oxidative stress induced by CP (Fig.4.1.6).

4.1.4 The *Allium cepa* root meristem assay- effect of apocynin on mitotic index and chromosomal aberrations induced by cyclophosphamide.

4.1.4.1 Mitotic index (MI)

The mitotic index was highly decreased in the onion root meristems by treatment with CP (positive control/drug control) as compared to the negative control (distilled water). Apocynin alone did not cause significant decrease in the mitotic index. Exposure of onion bulb rootlets to apocynin along with CP simultaneously caused a significant restoration of the mitotic index compared to CP alone (Table 4.2.3;Figure 4.1.7).

4.1.4.2 Frequency of chromosomal aberrations (fCA)

The frequency of CA was found to be appreciably very high in the clastogenic control as compared to plain drinking water. The action of CP was significantly reversed by inclusion of apocynin at concentrations of 100µg/ml and 200µg/ml along with CP. The different types of chromosomal aberrations identified and counted include disturbed metaphases, lagging chromosomes in anaphase, micronuclei, lose of chromosomes, anaphasic bridges and sticky nuclei (Figure 4.1.9 and Figure 4.1.10). Apocynin significantly

lowered the action of CP, causing a dose dependent reduction in the fCA, with 200µg/ml of apocynin being more effective than 100µg/ml. Apocynin by itself, at concentrations of 100µg/ml and 200µg/ml, did not induce significant number of aberrations (Table 4.2.3;Figure 4.1.8).

4.1.5 Study of cytotoxic and anticytotoxic properties of apocynin by MTT assay

4.1.5.1 Effect of apocynin and cisplatin on CHO-K1 and HepG2 cell lines and calculation of IC50 value

Apocynin showed very little cytotoxicity on both the cell lines CHO-K1 and HepG2 at all the tested concentrations (Table 4.1.4). Since the inhibition shown by apocynin is not significant the IC50 value was not calculated. Cisplatin showed significant toxicity on the cancer cells HepG2 as well as CHO-K1 (Table 4.1.5). The IC50 value of cisplatin was calculated using a dose response curve (Figure 4.1.11). The concentration of 30µg of cisplatin was chosen for further studies.

4.1.5.2 The effect of combination treatments (apocynin + cisplatin) on the cell lines

The effect of presence of apocynin on cisplatin induced growth inhibition was studied by treating the cells with three different proportions of apocynin and cisplatin simultaneously followed by MTT. The assay was performed first in single assays (Table 4.1.6 and Table 4.1.7 and in triplicates (Table 4.1.8). All the combination treatments showed a noteworthy inhibition of cell growth than that in the control which was considered as zero inhibition. The results show that apocynin lowered the inhibition caused by cisplatin very significantly in both CHO-K1 and HepG2 cells (Figure 4.1.12 and Figure 4.1.13). In both cases, the presence of apocynin, even at a concentration lower than cisplatin also quite significantly lowers the inhibition of cell growth as compared to cisplatin alone, although it is less than the protection by higher concentrations of apocynin.

A combination of Apo: Cis in 1:1 proportion showed the highest protection of both the cultures. In case of CHO-K1, the difference in protection offered by different proportions of apocynin against the same concentration of cisplatin is significant, where as in case of HepG2 apocynin did not show a dose dependent relation with protection against cisplatin induced cytotoxicity.

4.1.6 Influence of apocynin on cisplatin induced DNA fragmentation

The effect of cisplatin, apocynin and combination treatments on the fragmentation of DNA in cultured CHO-K1 cells was studied by extracting the genomic DNA and running it on agarose gel, in separate lanes along with a ladder of standard DNA. Fragmented DNA will run on the gel forming a smear of DNA, whereas intact DNA will remain as a distinct band. DNA from untreated cells is used as a control. CHO-K1 cells treated with cisplatin 30μM showed complete fragmentation in comparison with DNA from control cells. The cells treated with cisplatin 30μM + apocynin30μM and cisplatin 30μM + apocynin60μM did not show any fragmentation of DNA (Figure 4.1.14). Overall, these results suggest that cisplatin alone leads to fragmentation of DNA; however upon co-treatment, apocynin has rescued DNA from chromatin fragmentation caused by cisplatin.

4.1.7 Influence of apocynin on haemolysis

The influence of treatment with apocynin and cisplatin, in three different proportions of 1:1, 1:2 and 2:1(apocynin: cisplatin) on induction of haemolysis was studied and the results are presented in Table 4.1.9. None of the treatments showed any enhancement of lysis of erythrocytes as compared to the negative control. The presence of apocynin along with cisplatin did not induce any haemolytic ability in cisplatin. Similarly presence of cisplatin did not induce any haemolytic ability in apocynin. They do not show any synergistic haemolysis, in the studied combinations and concentrations (Fig.4.1.15).

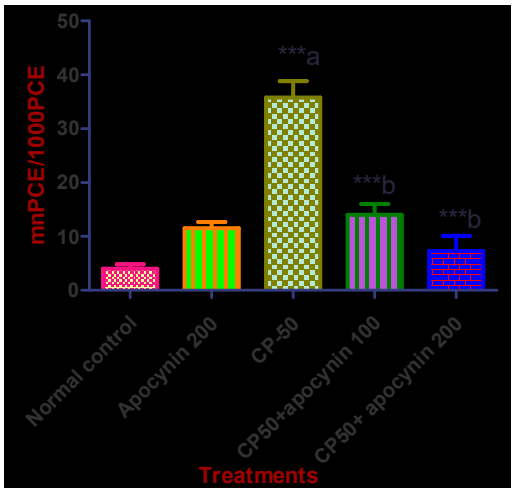


Figure 4.1.1 Influence of apocynin and CP on induction of MnPCEs in mice bone marrow

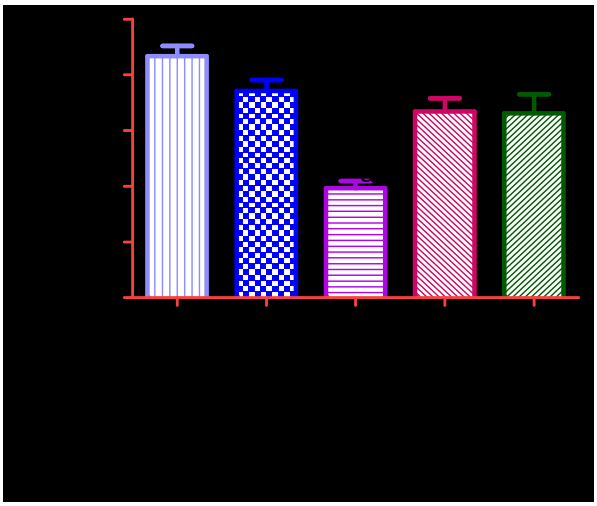


Figure 4.1.2 Influence of apocynin and CP on total WBC count in mice

a –when compared with normal control
b –when compared with clastogenic or positive control
CP =Cyclophosphamide; Apo =Apocynin [*P<0.05, **P<0.01, ***P<0.001

Table 4.1.1

Effect of apocynin on CP induced genotoxic effects in mice

Treatment	Mean No. of MnPCE/1000 PCE	WBC count (cells/cu.mm)	RBC count (million cells/cu.mm)	Haemoglobin content (µg/cu.mm)
Normal control	4.00 ± 0.81	866 ± 366.9	7.23 ± 0.46	12.61 ± 0.310
Apocynin 200µg/kg (p.o.)	11.50 ±1.19	7418 ± 390.9	7.78±0.43	13.69±0.864
CP50mg/kg (i.p.) [clastogenic or positive control]	35.75±3.0^{***a}	3928±257.2^{***a}	7.615±0.33	10.56±1.163
CP50mg/kg +Apo100 µg/kg	14.00±2.0^{**b}	6676±476.3^{**b}	7.465±0.33	8.708±0.789
CP50mg/kg +Apo200 µg/kg	7.25±2.8^{***b}	6615±678.2^{**b}	8.513±0.35	10.75±0.263

Table 4.1.2 Effect of apocynin on LPO and GSH levels in experimental animals

Treatment	LPO (nmol/gm wet tissue)	GSH (nmol/gm wet tissue)
Normal control	3.10±0.62	35.21±3.56
Apocynin 200µg/kg (p.o.)	5.30±0.26	29.21±2.87
CP50mg/kg (i.p.) [clastogenic or positive control]	20.32±4.10^{***a}	10.25±3.65^{***a}
CP50mg/kg +Apo100 µg/kg	11.16±3.15^{*b}	22.15±2.32^{*b}
CP50mg/kg +Apo200 µg/kg	8.32±2.52^{**b}	28.16±4.12^{**b}

a –when compared with normal control

b –when compared with clastogenic or positive control

CP =Cyclophosphamide; Apo =Apocynin [Values expressed are mean + SEM, n-06]

*P<0.05, **P<0.01, ***P<0.001

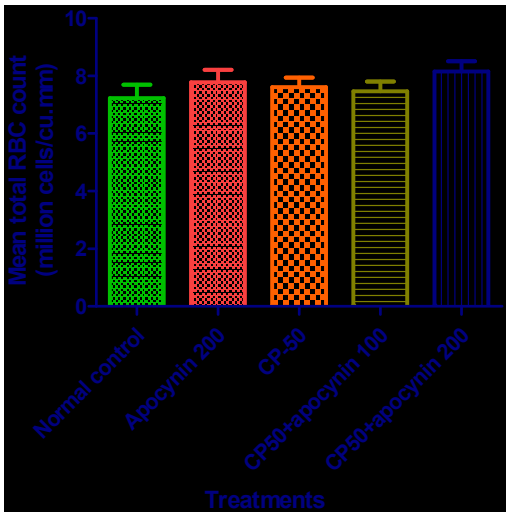


Figure 4.1.3
Influence of apocynin and CP on total RBC count in mice

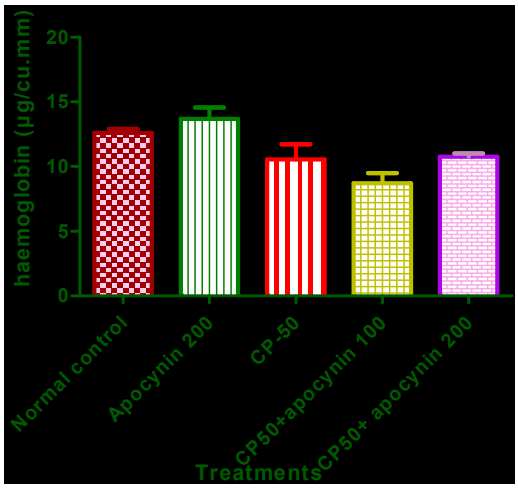


Figure 4.1.4
Influence of apocynin and CP on haemoglobin content in mice
a –when compared with normal control b –when compared with clastogenic or positive control CP =Cyclophosphamide; Apo =Apocynin

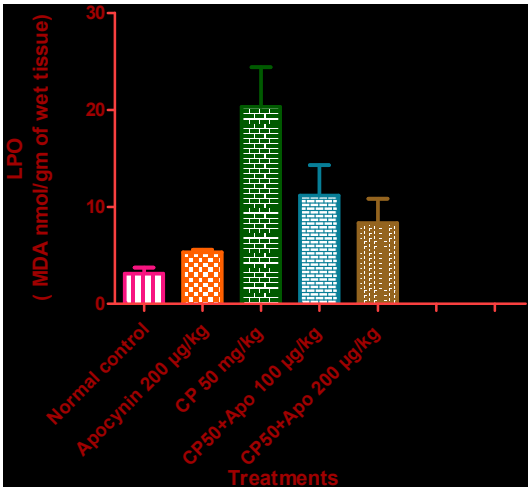


Figure 4.1.5 Influence of apocynin and CP on lipid peroxidation in mice

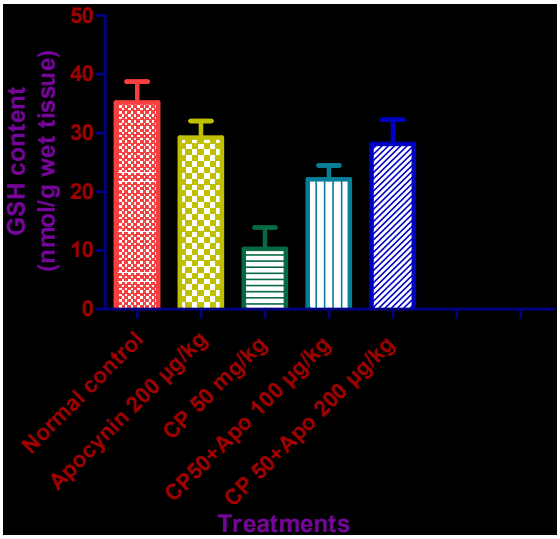


Figure 4.1.6 Influence of apocynin and CP on GSH content

a –when compared with normal control

b –when compared with clastogenic or positive control

CP =Cyclophosphamide; Apo =Apocynin *P<0.05, **P<0.01, ***P<0.001

Table 4.1.3 MI and Frequency of CA in *Allium cepa* root meristem assay

Group	Treatment (n=3)	Mean ± SEM of MI	Mean±SEM of fCA
1	Negative control (plain drinking water)	19.33±2.322	0.00±0.00
2	positive control (CP 2mg/ml in water)	3.56±_0.266^{***a}	0.48±0.08^{***a}
3	CP 2mg/ml +Apocynin 100µg/ml in water	9.86±_0.792^{*b}	0.16±0.04^{*b}
4	CP 2mg/ml +Apocynin 200µg/ml in water	15.17±0.456^{***b}	0.10±0.07^{**b}
5	Apocynin 100µg/ml in water	16.83±1.084	0.08±0.01
6	Apocynin 200µg/ml in water	16.85±0.350	0.05±0.02

a–when compared with normal control
b–when compared with clastogenic or positive control
CP =Cyclophosphamide; Apo =Apocynin [Values expressed are mean + SEM, n-03]
*P<0.05, **P<0.01, ***P<0.001

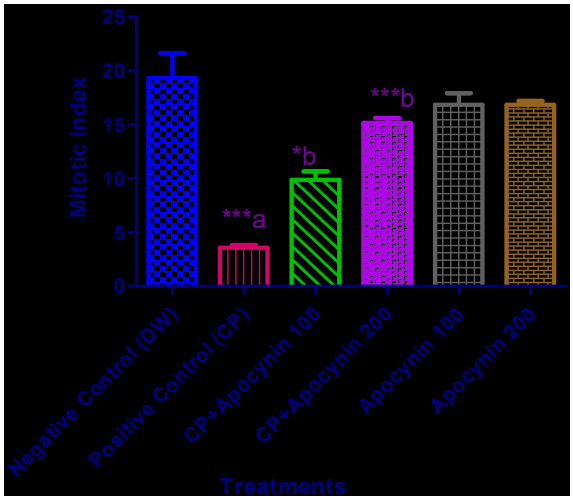


Figure 4.1.7 Influence of apocynin and CP on the MI in *Allium cepa* root meristems

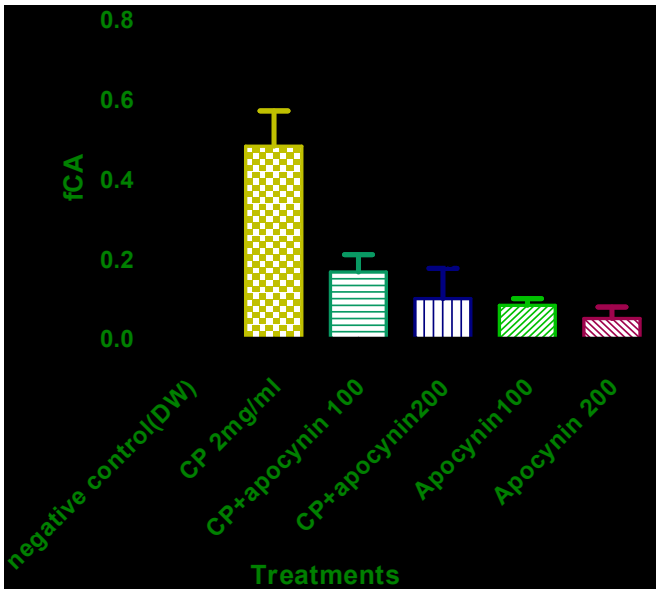
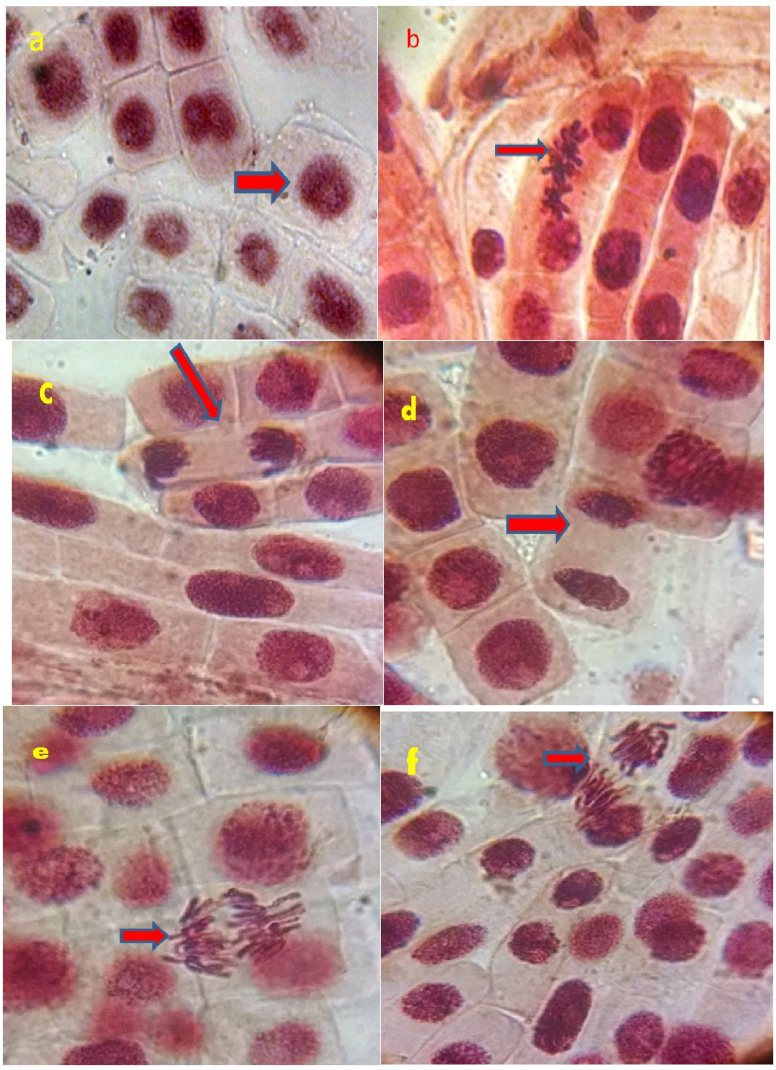


Figure 4.1.8 Influence of apocynin and CP on the CA in root meristems

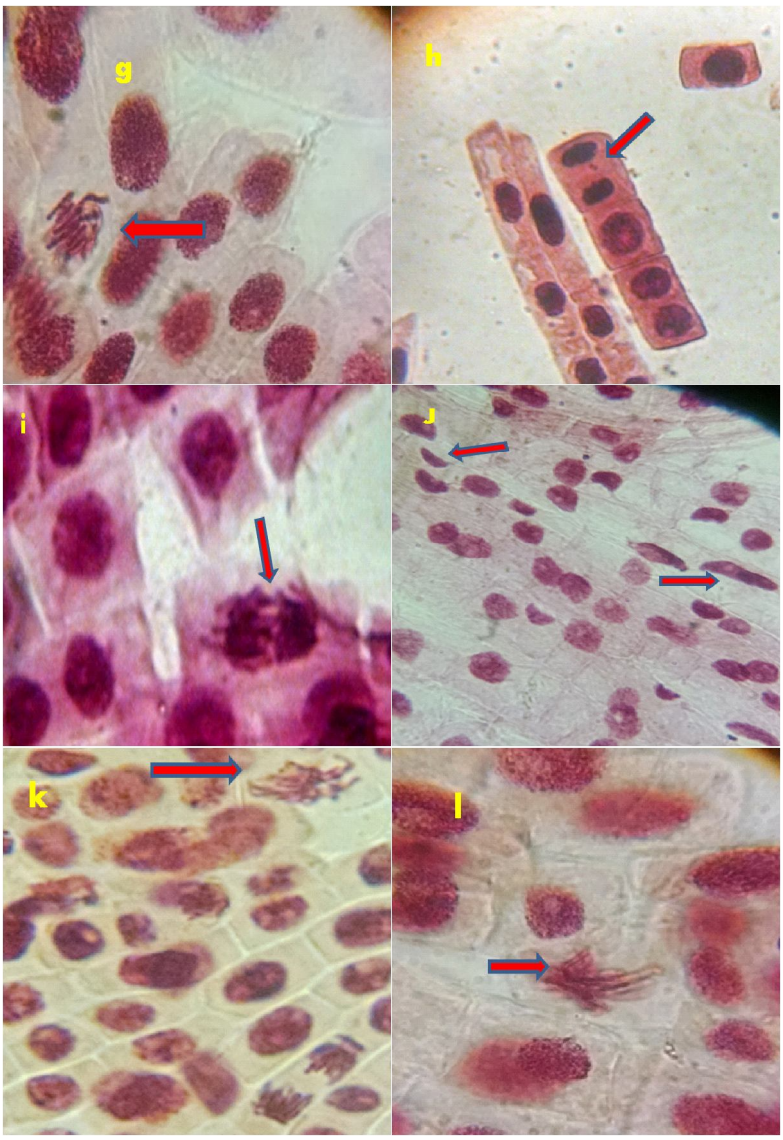
a–when compared with normal control
b–when compared with clastogenic or positive control
CP =Cyclophosphamide; Apo =Apocynin
*P<0.05, **P<0.01, ***P<0.001

cont...



a,b,c,d – normal stages of mitosis — prophase, metaphase, anaphase and telophase;
e and f – Mitotic abnormalities — lagging chromosomes in anaphase

Figure 4.1.9 Different types of mitotic stages



Mitotic abnormalities – g -metaphase with lose of chromosomes; h – micronuclei in telophase; i – anaphasic bridges; j – sticky nucleus; k and l – disturbed metaphase

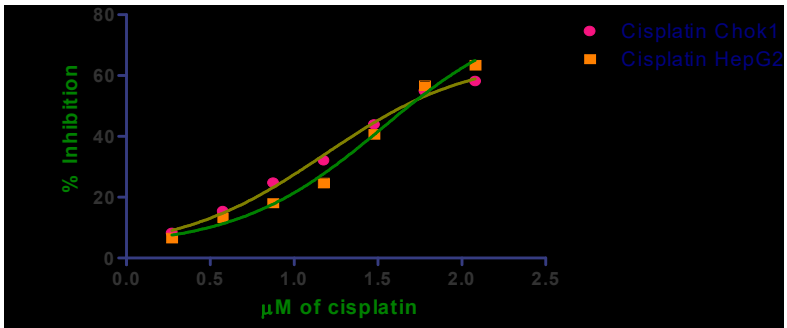
Figure 4.1.10 Different types of mitotic aberrations

Table 4.1.4 The effect of apocynin on growth of CHO-K1 and HepG2 cells in culture

10000 cells/well	Conc. of Apocynin(µM)	OD at 590 nm	%Inhibition	IC50 of Apocynin(µM)
CHO-K1	Control	0.487	00.00	NA
	1.87	0.480	01.44	
	3.75	0.453	06.98	
	7.5	0.445	08.62	
	15	0.421	13.55	
	30	0.408	16.22	
	60	0.402	17.45	
	120	0.399	18.07	
HepG2	Control	0.499	00.00	NA
	1.87	0.488	02.20	
	3.75	0.465	06.81	
	7.5	0.455	08.82	
	15	0.432	13.43	
	30	0.417	16.43	
	60	0.401	19.64	
	120	0.389	22.04	

Table 4.1.5 The effect of cisplatin on growth of CHO-K1 and HepG2 cells in culture and calculation of IC 50 value

10000 cells/well	Conc. of cisplatin(µM)	OD at 590 nm	% Inhibition	IC50 of Cisplatin(µM)
CHO-K1	Control	0.473	00.00	15.23
	1.87	0.434	08.25	
	3.75	0.400	15.43	
	7.5	0.356	24.74	
	15	0.321	32.14	
	30	0.265	43.97	
	60	0.213	54.97	
	120	0.198	58.14	
HepG2	Control	0.488	00.00	33.65
	1.87	0.456	06.56	
	3.75	0.423	13.32	
	7.5	0.399	18.24	
	15	0.367	24.80	
	30	0.289	40.78	
	60	0.211	56.76	
	120	0.178	63.52	



	Cisplatin Chok1	Cisplatin HepG2
log(inhibitor) vs. response		
Best-fit values		
BOTTOM	66.15	82.35
TOP	2.093	3.382
LOGIC50	1.183	1.527
IC50	15.23	33.65

Figure 4.1.11
Dose response curve for calculation of IC50 value of cisplatin

Table 4.1.6
Effect of apocynin combination treatments on CHO-K1 cell line

CHO-K1			
S.No.	**Treatment**	**OD at 590nm**	**% Inhibition**
1	Control	0.411	0
2	Apo (30µM):Cis (30µM) - 1:1	0.332	19.20
3	Apo (30µM):Cis (60µM) - 1:2	0.254	38.23
4	Apo (60µM):Cis (30µM) - 2:1	0.302	26.50

Table 4.1.7
Effect of apocynin combination treatments on HepG2 Cell line

HepG2			
S.No.	**Treatment**	**OD at 590nm**	**% Inhibition**
1	Control	0.445	0
2	Apo (30µM): Cis(30µM) - 1:1	0.336	24.40
3	Apo (30µM): Cis(60µM) - 1:2	0.278	37.49
4	Apo (60µM): Cis(30µM) - 2:1	0.322	27.59

Table 4.1.8 Effect of combination treatments on CHO-K1 and HepG2 (triplicates)

	CHO-K1			HepG2			
Treatment	**OD value at 590nm**	**% Inhibition**	**Mean±SE**	**Treatment**	**OD value at 590nm**	**% Inhibition**	**Mean±SE**
Control	0.453	00.00	0.0	**Control**	0.467	00.00	0.0
	0.443	00.00			0.489	00.00	
	0.463	00.00			0.476	00.00	
Cisplatin alone	0.243	46.35	48.50± 1.19^{***a}	**Cisplatin alone**	0.257	44.97	48.11± 3.02^{***a}
	0.221	50.11			0.224	54.19	
	0.236	49.03			0.261	45.16	
Apo (30µM): Cis(30µM) - 1:1	0.367	18.98	16.58± 2.20^{***b}	**Apo (30µM): Cis(30µM) - 1:1**	0.356	23.77	23.29± 0.67^{***b}
	0.389	12.18			0.377	22.90	
	0.377	18.57			0.356	25.21	
Apo (30µM): Cis(60µM) - 1:2	0.267	41.06	37.98± 1.61^{***b}	**Apo (30µM): Cis(60µM) - 1:2**	0.308	34.05	34.29± 0.19^{***b}
	0.278	37.25			0.322	34.15	
	0.298	35.64			0.311	34.66	
Apo (60µM): Cis(30µM) - 2:1	0.311	31.35	28.91± 1.24^{***b}	**Apo (60µM): Cis(30µM) - 2:1**	0.367	21.41	22.27± 0.43^{***b}
	0.322	27.31			0.378	22.70	
	0.333	28.07			0.368	22.70	

[Values expressed are mean + SEM, n-03]

* P<0.05, **P<0.01, *** P<0.001

a – when compared with normal control; b –when compared with cytotoxic or positive control-cisplatin; Apo= apocynin, Cis= Cisplatin

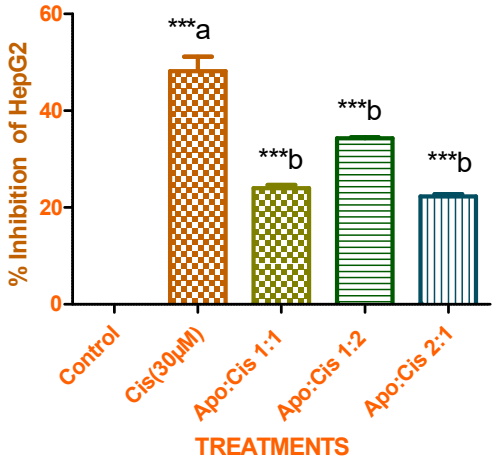


Figure 4.1.12
Effect of combination treatments on HepG2 cell line

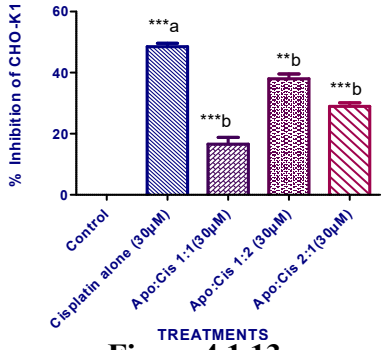
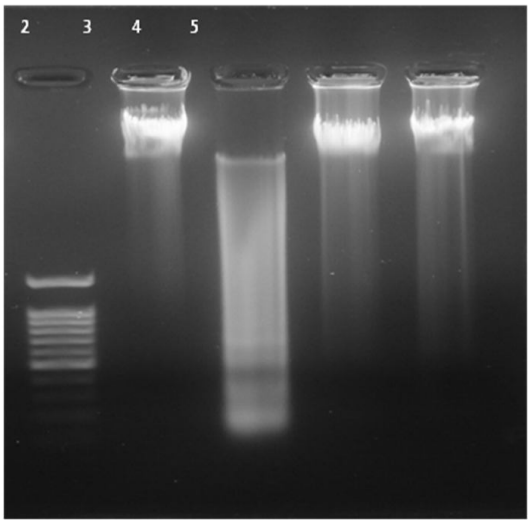


Figure 4.1.13

Effect of combination treatments on CHO-K1 cell line

a – when compared with normal control;

b –when compared with cytotoxic or positive control-cisplatin * P<0.05, **P<0.01, *** P<0.001; Apo= apocynin, Cis= cisplatin



Lane 1: 100bp ladder;
Lane 2: control
Lane 3: Cis30 µM
Lane 4: Apo30µM+Cis30µM
Lane 5 : Apo60µM+Cis30µM

Figure 4.1.14 Agarose gel electrophoresis of DNA extracted from CHO-K1 cells

Table 4.1.9 The effect of combinations of apocynin and cisplatin on haemolysis

S. No.	Treatment	Absorbance	% Inhibition
1	Negative Control (PBS)	0.791	0.00
2	Positive control – 1% SDS	0.187	76.37
3	Apocynin(30 µM) : Cisplatin(30 µM)1:1	0.617	22.07
4	Apocynin(30 µM) : Cisplatin(60 µM) 1:2	0.561	29.04
5	Apocynin(30 µM) : Cisplatin(60 µM) 2:1	0.570	27.93

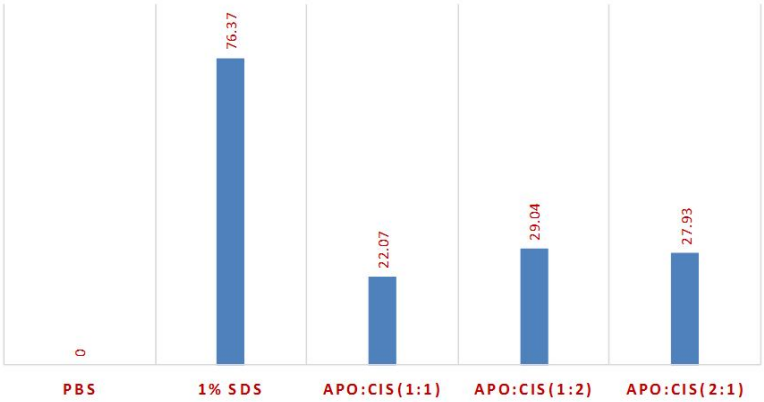


Figure 4.1.15 Effect of apocynin and cisplatin on haemolysis

4.2 Genotoxic and Antigenotoxic Effects of Diosgenin

4.2.1 Influence of diosgenin on induction of MnPCEs in mice bone marrow

Results obtained after the various groups of experimental animals are subjected to treatments are summarized in Table 4.2.1.The intraperitoneal treatment of mice with CP at the rate of 50mg/kg bodyweight resulted in a statistically significant amplification of the number of MnPCE's per 1000 PCE than in the normal control. Treatment with diosgenin alone, orally, did not raise significantly the number of MnPCEs. In mice treated with

CP50+Diosgenin 100 and CP50+Diogenin 200mg/kg body weight, the MnPCEs were significantly reduced as compared to treatment with CP50 alone. The effect of diosgenin pretreatment was dose dependent, with the higher dosage of diosgenin being more effective in lowering the mnPCE frequency (Figure 4.2.1).

4.2.2 Effect of diosgenin on haematological parameters in experimental animals

The effects of treatments on the blood parameters namely total count of WBC, RBC and HB were analysed and the results of these experiments are presented in Table 4.2.1.

4.2.2.1 Total WBC count

CP administration leads to significant decline of total leucocytes as compared to normal control group of mice. Though administration of diosgenin alone caused a insignificant drop in the total WBC count than in normal control, it was very effective in replenishing the WBC loss caused by CP in a significant manner (Figure 4.2.2).

4.2.2.2 Total Erythrocyte count and Haemoglobin percentage

None of the treatments namely diosgenin200 µg/kg body weight, CP50mg/kg and the combinations of CP with 100 µg/kg or 200 µg/kg diosgenin, significantly altered the total count of RBC or haemoglobin content compared to normal control (Figure 4.2.3 and Figure 4.2.4).

4.2.3 Effect of diosgenin on oxidative stress parameters

The results of the study of oxidative stress parameters namely levels LPO and GSH under the influence of the various treatments are presented in Table 4.2.2.

4.2.3.1 Effects of diosgenin on the level of lipid peroxidation

We estimated the level of LPO in the liver using MDA as

an indicator. The results indicated a significant boost in the lipid peroxidation in liver tissue of animals exposed to CP compared to control. In animals pre-treated with diosgenin, there was a significant lowering of the CP induced lipid peroxidation (Figure 4.2.5).

4.2.3.2 Estimation of the level of reduced glutathione

Ability of diosgenin in countering the oxidative stress induced by CP was evidenced by the pattern of cellular antioxidant GSH under the influence of the treatments. CP caused a considerable fall in the cellular GSH levels. On the other hand, the same was restored by diosgenin pre-treatment (Figure 4.2.6). At the same time, diosgenin *per se* did not cut down the hepatic GSH levels, which is an indication of absence of oxidative stress induction.

4.2.4 Study of cytotoxic and anti- cytotoxic properties of diosgenin by MTT assay

4.2.4.1 Effect of diosgenin on CHO-K1 and HepG2 cell lines and calculation of IC50 value

Diosgenin did not have significant cytotoxicity on CHO-K1 cells at all the tested concentrations (Table 4.2.3). Henceforth the IC50 value for diosgenin on CHO-K1 cells was not possible to calculate. Diosgenin showed significant cytotoxic influence on HepG2 cells (Table.4.2.3) and the IC50 value of 84.4 µM was obtained (Figure 4.2.7) from the dose response curve. Three concentrations of diosgenin 25µM, 50µM and 100µM were chosen for study of the combined effect of cisplatin and diosgenin on both the cells. The cisplatin concentration was chosen as 30 µM for the combination treatments.

4.2.4.2 The effect of combination treatments (diosgenin + cisplatin) on the cell lines

The outcome of addition of three different concentrations of diosgenin on cisplatin induced cell inhibition was studied by MTT

assay first in individual sets (Table 4.2.4 and Table 4.2.5) and then in triplicates. The results of triplicate studies are presented in Table 4.2.6.

Diosgenin showed a significant enhancement of the growth inhibition induced by cisplatin on HepG2 cells, at all the three tested combinations compared to the control which is considered as zero inhibition (Table.4.2.6). The synergistic enhancement of cytotoxicity of a cisplatin was dose dependent, following the increase in the concentration of diosgenin (Figure 4.2.9).

In case of CHO-K1 cells the simultaneous treatment of cells with diosgenin and cisplatin did not show any enhancement of cytotoxicity of cisplatin. (Table 4.2.6). Also there was no prominent decrease in the cytotoxicity of cisplatin towards the CHO-K1 cells at any of the combinations of diosgenin and cisplatin studied (Figure 4.2.8).

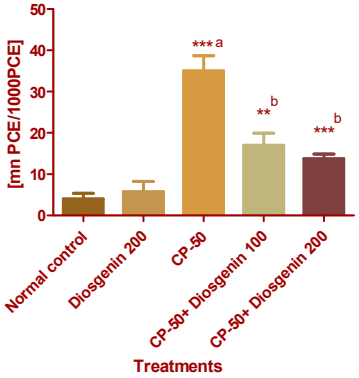


Figure 4.2.1 Effect of diosgenin treatments on frequency of mnPCEs

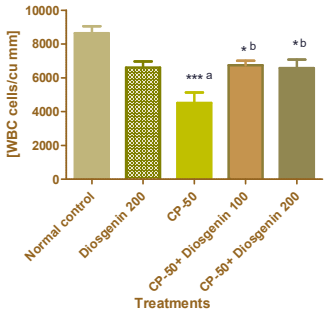


Figure 4.2.2 Effect of diosgenin treatments on total WBC count

a –when compared with normal control b –when compared with clastogenic or positive control CP =Cyclophosphamide; *P<0.05, **P<0.01, ***P<0.001

Table 4.2.1
Influence of diosgenin on genotoxic effects of CP in mice

Treatment	Mean No. of MnPCE/1000 PCE	WBC (cells/cu.mm)	RBC (million cells/cu.mm)	Haemoglobin (µg/cu.mm)
Normal control	4.00±1.35	8640±403.0	7.35±0.38	12.68±0.31
Diosgenin 200µg/kg (p.o.)	5.75±2.44	6603±355.6	7.15±0.38	12.00±1.27
CP50mg/kg (i.p.) [clastogenic/ positive control]	35.00±3.69***a	4511±618.4***a	7.16±0.48	10.66±1.09
CP50mg/kg +Dio100 µg/kg	17.00±2.94**b	6739±268.9*b	7.79±0.29	14.14±0.86
CP50mg/kg +Dio200 µg/kg	13.75±1.10***b	6574±499.6*b	7.058±0.6	13.04±1.77

Table 4.2.2
Effect of the diosgenin on oxidative stress parameters in mice

Treatment	LPO (nmol/gm wet tissue)	GSH (nmol/gm wet tissue)
Normal control	2.29± 0.19	14.83±1.76
Diosgenin (200 µg /kg p.o.)	1.21± 0.61	12.46±1.65
CP 50mg/kg i.p.	9.02± 2.15**a	2.51±2.16***a
CP 50mg+Dios 100 µg/kg p.o.	5.21±0.94*b	8.42±2.32*b
CP50mg+Dios 200 mg/kg p.o.	4.05±0.58*b	10.54±3.16**b

a –when compared with normal control
b –when compared with clastogenic or positive control
CP =Cyclophosphamide;
[Values expressed are mean + SEM, n-06]

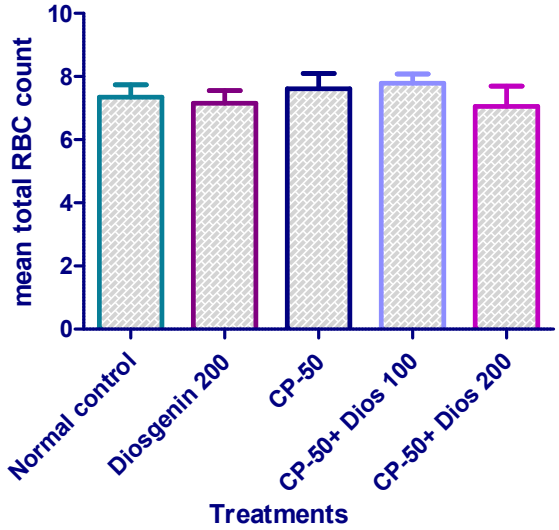


Figure 4.2.3 Effect of the treatments on total count of RBC

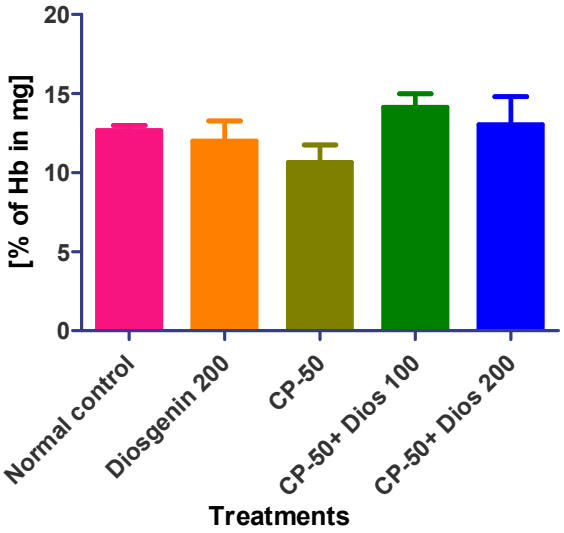


Figure 4.2.4 Effect of diosgenin treatments on haemoglobin content in mice

a –when compared with normal control b –when compared with clastogenic or positive control

CP =Cyclophosphamide

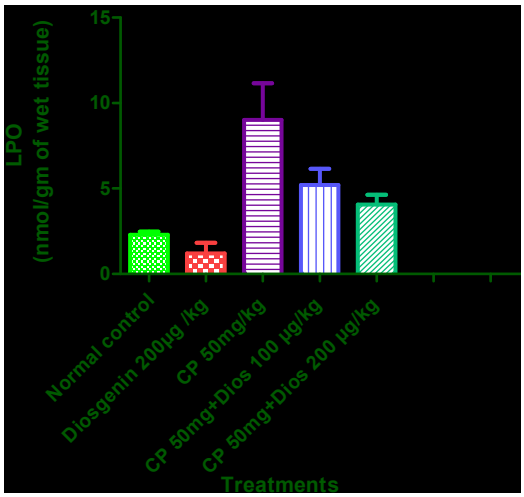


Figure 4.2.5

Influence of diosgenin and CP on lipid peroxidation in mice

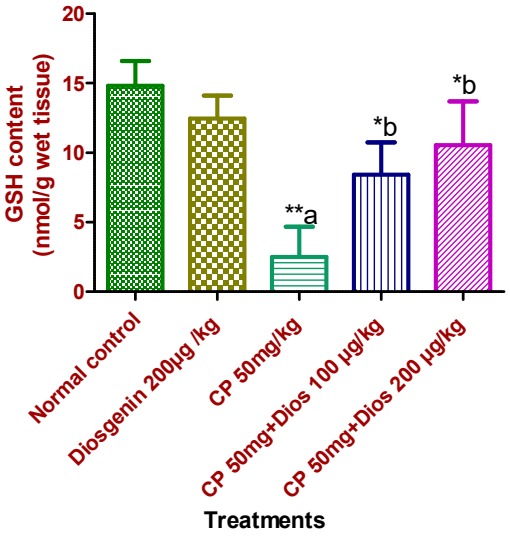


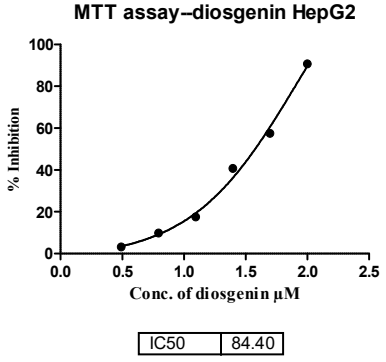
Figure 4.2.6

Effect of diosgenin treatments on GSH level in mice

a –when compared with normal control b –when compared with clastogenic or positive control CP =Cyclophosphamide; *P<0.05, **P<0.01, ***P<0.001

Table 4.2.3 Effect of diosgenin on the growth of CHO-K1 and HepG2 cells and calculation of IC 50 value

10000 cells/well	Conc. Of diosgenin(µM)	OD at 590 nm	% Inhibition	IC50 of diosgenin (µM)
CHO-K1	Control	0.647	0.00	NA
	3.12	0.627	3.05	
	6.25	0.601	6.98	
	12.5	0.571	11.65	
	25	0.518	19.88	
	50	0.488	24.55	
	100	0.434	32.83	
HepG2	Control	0.658	0.00	84.4 µM
	3.12	0.637	3.14	
	6.25	0.594	9.67	
	12.5	0.543	17.47	
	25	0.391	40.60	
	50	0.280	57.37	
	100	0.062	90.59	



log(inhibitor) vs. response	
Best-fit values	
Bottom	167.5
Top	-2.523
LogIC50	1.926
IC50	84.40

Figure 4.2.7 Dose response curve for calculation of IC50 value of diosgenin on HepG2 cells

Table 4.2.4 Effect of combinations of diosgenin and cisplatin on CHO-K1 cells

S. No.	Treatment	O.D at 590 nm	% Inhibition
1	Control	0.645	0.00
2	Diosgenin 25µM +cis 30µM	0.375	41.89
3	Diosgenin 50µM +cis 30µM	0.384	43.47
4	Diosgenin 100µM +cis 30µM	0.370	42.62

Table 4.2.5 Effect of combinations of diosgenin and cisplatin on HepG2 cells

S. No.	Treatment	O.D at 590 nm	% Inhibition
1	Control	0.660	0.00
2	Diosgenin 25µM +cis 30µM	0.328	50.32
3	Diosgenin 50µM +cis 30µM	0.230	75.12
4	Diosgenin 100µM +cis 30µM	0.197	95.13

Table 4.2.6 Effect of diosgenin and cisplatin on HepG2 cells and CHO-K1 (in triplicates)

HepG2				CHO-K1			
Treatment	OD value at 590nm	% inhibition	Mean±SE	Treatment	OD value at 590nm	% inhibition	Mean±SE
Control	0.657	0	0	**Control**	0.647	0	0
	0.662	0			0.660	0	
	0.649	0			0.628	0	
Cisplatin alone30µM	0.377	43.35		**Cisplatin alone30µM**	0.356	44.97	44.79± 0.296*** a
	0.363	45.11	43.50± 0.892*** a		0.368	44.19	
	0.376	42.03			0.344	45.16	
Diosgenin 25µM +Cis (30µM)	0.305	54.32	54.22± 0.804*** b	**Diosgenin 25µM +Cis (30µM)**	0.356	41.45	42.00± 1.153*** a
	0.312	52.78			0.368	44.21	
	0.288	55.56			0.344	40.33	
Diosgenin 50µM +Cis (30µM)	0.232	65.12	65.10± 1.4*** b	**Diosgenin 50 µM +Cis (30µM)**	0.378	40.73	42.03± 1.133*** a
	0.215	67.51			0.368	44.29	
	0.242	62.66			0.374	41.08	
Diosgenin 100µM +Cis (30µM)	0.065	90.13	93.88± 1.89*** b	**Diosgenin 100 µM +Cis (30µM)**	0.371	42.62	44.99± 2.02*** a
	0.031	95.28			0.374	43.34	
	0.024	96.22			0.320	49.01	

a – when compared with normal control

b –when compared with cytotoxic or positive control

[Values expressed are mean + SEM, n-03]

* P<0.05, **P<0.01, *** P<0.001 Cis =Cisplatin

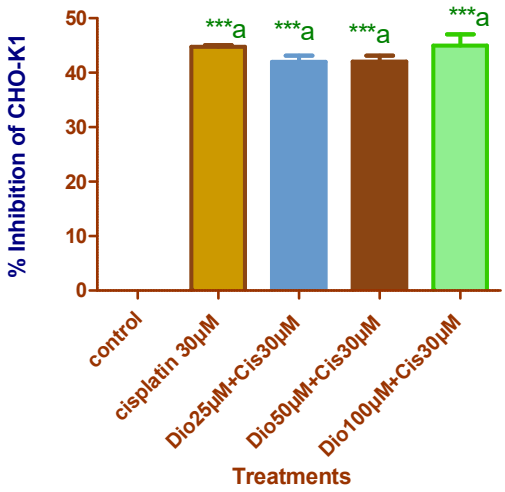


Figure 4.2.8 Effect of combinations of diosgenin and cisplatin on CHO-K1 cells

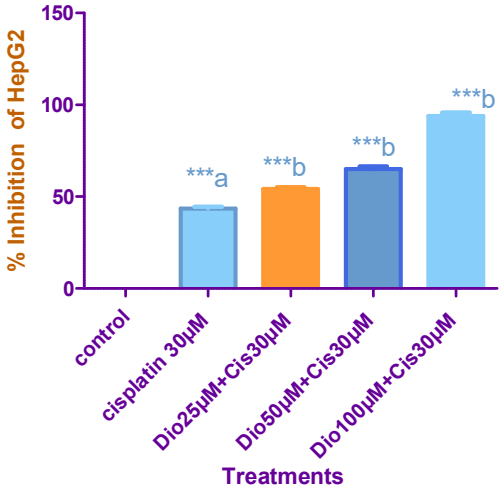


Figure 4.2.9 Effect of combinations of diosgenin and cisplatin on HepG2 cells

[Values expressed are mean + SEM, n-03]

* P<0.05, **P<0.01, *** P<0.001

a – when compared with normal control;

b –when compared with cytotoxic or positive control-cisplatin

CHAPTER - 5

The current investigation was taken up with the objective of studying the genotoxic and antigenotoxic properties of plant compounds apocynin and diosgenin. The chemotherapy drugs cyclophosphamide and cisplatin were employed as positive controls or drug controls to induce genotoxic and cytotoxic conditions in the experimental procedures. The genotoxic and cytotoxic effects of apocynin and diosgenin observed in the different *in vivo* as well as *in vitro* assays and the possible mechanisms responsible for the observed results are discussed in this chapter.

5.1 Genotoxic and Antigenotoxic Influences of Apocynin

The capacity of apocynin to amend the genotoxic damage inflicted by CP was investigated using the mouse bone marrow erythrocyte micro nucleation assay, assessment of haematological parameters, evaluation of the oxidative stress biomarkers namely lipid peroxidation and reduced glutathione and *Allium cepa* root meristem chromosomal aberrations assay. The cytotoxic and

anticytotoxic abilities were assessed by performing cell line growth inhibition test *in vitro*.

The mouse erythropoietic system is a highly suitable *in vivo* system which allows the evaluation of antigenotoxicity and erythropoietic safety of any biomaterial that has therapeutic value, by interpreting the frequency of micronuclei in polychromatic erythrocytes of bone marrow [116]. Occurrence of micronuclei is a consequence of genotoxic insult to the chromosomes.

CP is recommended as a standard agent to induce genotoxicity as per OECD guide lines [27,54]. Cyclophosphamide is a nitrogen mustard alkylating agent from the oxazophorines group and is also known as cytophosphane. It alkylates the 7^{th} nitrogen atom in the imidazole structure of guanine [63] and forms DNA crosslinks, within, as well as between the strands. Cells with cross-linked DNA are incapable of cell division and subsequently die. CP is used in the management of lymphomas, leukaemia, tumours of the brain and other tissues [39] and also to produce immunosuppressive effects [192]. It is bio activated in the liver by cytochrome P-450 oxidase system to its reactive electrophilic metabolites phosphoramide mustard and acrolein. The slow accumulation of these activated molecules in the plasma, leads to severe cytotoxic effects in non target tissues also [171]. Phosphoramide mustard is the component which causes alkylation of DNA strands. Several analyses have shown CP to disrupt the redox homeostasis of normal tissues indicating that oxidative stress is the basis of physiological and biochemical damages caused by it [15,65]. In the current study, CP induced chromatin damage was evident by spiking of the frequency of MnPCE in mice bone marrow following an exposure to CP, at the rate of 50mg/kg of the experimental animals.

Apocynin showed marked reduction of micronucleation in

the PCE's of mice exposed to CP in the present investigation, when administered in combination treatments along with CP, in a dose dependent pattern. The higher dose of 200µg/kg body weight was more efficient. Available experimental literature by different authors indicates that CP induced geno - and cyto-toxicity may be countered by activity of quite a number of natural and synthetic antioxidants [115, 65, 27] deliver significant anticlastogenic effects against CP, as reflected by reduction in a array of biological indicators of genotoxic damage.

Plant polyphenols contribute several such agents capable of protecting DNA from genotoxic damage inflicted by CP and other chemotherapeutics also [13, 26, 28, 48, 64]. Apocynin, the molecule under investigation in the current study is a phenolic substance, already well known for its other potent therapeutic applications. The information related to its genotoxic and antigenotoxic nature was scarcely available. The antigenotoxic nature of apocynin was clearly evident from the results of the micronucleus test in our study. It is vital to evaluate the direct effect of these plant constituents, on the genetic material. In the present study apocynin was found to be non genotoxic to the cells. Further, it clearly did not show any synergistic effects with CP in causing genotoxicity. Earlier literature also supports that apocynin lacked any genotoxicity in Ames test and SCE-test as well [193].

Several different mechanisms are said to be involved in the antigenotoxic nature of plant compounds. Phytochemicals can be incorporated in to chemoprevention systems, by virtue of their direct antioxidant mechanisms or indirect modulation of enzyme activities [143]. Grape fruit juice may function as a catalytic inhibitor of topoisomerase II and also elicits phase II enzymes responsible detoxification [64]. A probable mode of antigenotoxic activity of cimetidine against benzene is reversible inhibition of cytochrome P-450 obstructing the conversion of toxins into their active

metabolites [7]. Phenolic compounds are also capable of boosting the activity of detoxifying phase I enzymes besides suppressing the phase II enzymes implicated in the xenobiotic activation [13, 115]. They can even amend the DNA rescue and repair machinery along with hindering the formation of DNA adducts or methylation, by blocking the electrophilic site(s) in the DNA, which are labile to assault by reactive species [7, 114].

The major mechanism of action of apocynin in being a powerful antioxidant and anti inflammatory agent is blocking of NADPH oxidase [9]. Other mechanisms are also proposed for the anti-inflammatory action of apocynin by different authors [76]. Apocynin is a effective inhibitor of NAT enzymes related with a broad range of cancers and projected as possible drug targets [143]. It is also reported that in nonphagocytic cells apocynin principally acts as a reducer of ROS and subsequent ROS-induced signalling [9] rather than inhibiting NADPH oxidases. In osteoblasts, apocynin, by activation of factors PI3K and CREB, appreciably reduced oxidative mitochondrial dysfunction induced by Antimycin-A [62]. In other tissues like endothelium, it inhibits cytochrome P450 activity, and in PMN, affects polymerization of actin and on lipid metabolism [9]. In the current study, reduction in the frequency of micronuclei is an indicator of the ability of apocynin to shield the cells from DNA injury. The possible mechanism behind this action of apocynin to protect DNA is by scavenging of free radicals, whose production is known to be greatly spiked by CP. Another highly possible reason may be inhibition of cytochrome p- 450 by apocynin, which in turn can prevent activation of CP into its toxic metabolite, which are actually responsible for generation of DNA breakage. Since there is a strong correlation between genomic damage and carcinogenesis, substances that prevent genotoxic insult to cells are potential chemopreventive agents [15, 26, 102]. Understanding the effect of potent therapeutic molecules on haematological parameters like leucocytes, erythrocytes and HB

forms a key component in further making sure their compatibility with the biological system. Chemotherapy agents like CP, CCNU and 5-FU are known to cause a significant reduction in the total lymphocyte as well as total WBC counts [114, 171, 194]. Cyclophosphamide weakens both the cell-mediated and humoral immune response in a dose-dependent manner, causing immunosuppression [192, 195]. Phosphoramide mustard is the major causative factor for this loss of immune function. This makes the individual susceptible to various types of secondary infections [171]. In the present study also, CP caused a significant depression in total WBC count where as no significant influence of CP and apocynin on HB content and RBC count could be observed in the current study. Apocynin could alleviate the leucopenia induced by CP in mice. Studies by other workers with natural extracts have shown comparable protective/ restorative consequence on CP-induced WBC depression [196,197] .The results indicate that apocynin could stimulate the haemopoetic system and have immunomodulatory effects. The exact mechanism of apocynin in the amelioration of CP induced WBC loss needs to be further explored. One possible explanation is non conversion of CP into phosphoramide mustard; Apocynin is a potent inhibitor of cytochrome system needed for this conversion.

We also evaluated the prophylactic effect of apocynin on the oxidative stress induced by CP. Measurement of lipid peroxidation is a vastly accepted indicator of oxidative turbulence. The positive association between degree of lipid peroxidation and genotoxicity as has been verified by many researchers [6, 198].

In the current study, the administration of CP enhanced LPO significantly compared to the normal control. Acrolein, one of the CP metabolites is responsible for the diminution of nucleophilic antioxidants, such as glutathione [199].

Exposure to apocynin alone did not enhance the LPO in the hepatic tissues in the current study. Further, priming of experimental animals with apocynin significantly reduced the CP induced lipid peroxidation in the liver. Several experimental results support this observation. Dietary inclusion of natural antioxidants like EPA [65] and *Garcinia indica* fruit extract [82] prevented lipid peroxidation in tissues of experimental animals. Apocynin also was shown to boost the antioxidative defence system by increasing the activity of enzymes like SOD, GSHpx and GSH [76]. Earlier studies have also shown apocynin to normalize oxidative DNA damage triggered by cisplatin in mice [79].

A major decline in the GSH content has frequently been observed in different tissues as a result of CP [80, 171, 199]. The results of our current study are in agreement with these, where in, the levels of GSH were lowered significantly by CP. Apocynin did not contribute the depletion of GSH; rather, we noticed a significant revival of hepatic GSH in the mice subjected to apocynin pretreatment. This enhancement of GSH by apocynin could be crucial in annulling the genotoxic effects of CP. GSH and GSH-inducing compounds were found important in the prevention of such peroxidative damage induced by CP [27, 171, 199]. Glutathione is supposed to have a dual role in protection against the cytotoxicity of CP. It minimizes formation of the toxic species phosphoramide mustard [199] and also can neutralize another of its metabolite, acrolein [200]. The exact system of action of apocynin warrants further examination.

The antigenotoxic capability of apocynin was also evidenced in another biological system namely the *Allium cepa* root tip meristems, in our current research. This test system has been widely used, either singly or as a part of a battery of tests [90, 93, 95] to assess the mutagenicity of several natural and synthetic compounds, for biological monitoring, investigation of

environmental pollution, as well as evaluation of cytotoxicity and anti-proliferative potentials of medicinal compounds. Two parameters of importance namely the MI and fCA were calculated as markers of geno- and antigenotoxic influences of the compounds.

A good mitotic index (also called growth index) reveals normal progression of cell cycle. It is considered an indicator of adequate cell multiplication for evaluating the cytotoxic and genotoxic action of diverse environmental or therapeutic agents. We observed a mitotic depression in root tips in contact with CP. CP was found to be a significant mitodepressant in mice bone marrow and in root meristems as well, by other researchers [29, 90, 92]. The results of the onion root meristem analysis under the different treatments in the current study indicate that apocynin shows no substantial depression of MI. In onion bulbs treated simultaneously with apocynin and CP, apocynin notably restored the MI. CA also are one of the results of genomic damage by free radicals. The frequencies of CA in the root tips showed significant reduction in combination of apocynin and CP as opposed to CP alone, in this analysis. More than a few other plant compounds were also revealed to have moderating effects against clastogenic substances using the *Allium* assay. Kumara *et al*, 2012 reported antigenotoxic effects of aqueous extract of seeds of broccoli on cell division in root tips against herbicide ciluron[89]. Curcumin revealed antimutagenic properties against sodium azide [92, 188]. One of the important reasons for the wide spread use of *Allium* assay to evaluate possible antimutagenic substances is the presence of cytochrome P450 in higher plants [201]. Apocynin has an inhibitory effect on cytochromeP-450 [143]. As such, suppression of cytochromeP-450 mediated activation of CP to its active metabolites in the roots treated with a combination of apocynin and CP might be the plausible mechanism for the protection afforded by apocynin, since formation of these metabolites is inevitable for CP- mediated nucleic acid strand breaks. Apocynin also showed a cell cycle stimulatory effect by restoring the mitotic index.

To further interpret the eligibility of apocynin as a possible chemopreventive or chemoprotective supplement by virtue of its antigenotoxic strength, we performed cell growth inhibition assays using the MTT method. This is an efficient and dependable colorimetric assay for viability of cells, employed by researchers to understand the cytotoxic and anticytotoxic properties of a wide range of substances which in turn can reveal their potential as chemotherapeutic or chemopreventive agents, as well as cytoprotective abilities against environmental carcinogens [98, 101, 102]. With the current emphasis towards research and development of natural molecules as medicines, it is important to generate information regarding the toxicity and efficacy of plants and plant compounds utilized to treat ailments in the ethno botanical systems. In the present investigation, two cell lines namely CHO-K1, a normal cell line and HepG2, a hepatic cancer cell line, were chosen to study the cytotoxic and anticytotoxic effects of apocynin, against cisplatin induced cytotoxicity. The study aimed at understanding if apocynin had any inherent cytotoxicity and whether it had any synergistic/antagonistic effects with cisplatin. It also aimed to study whether apocynin had any selectivity towards a normal cell or towards a cancerous cell line, in its effects.

Apocynin did not exhibit any cytotoxic action on both the cell lines employed in our research. Cytotoxicity is the property of a substance to cause cell growth inhibition or death. The current study uses cisplatin [*cis*-Diaminedichloroplatinum(II)], a chemotherapeutic drug to induce cytotoxic conditions. Cisplatin is recommended for managing cancer of the testes, ovaries, cervix, brain, neck and the lungs in mono- or multi-drug regimen. It kills cancer cells by promoting DNA damage and inhibiting its synthesis. Interstrand and intrastrand cross-links generated in this process provide adjacent deoxyguanosines, which are the target sites for platination [86] by cisplatin. The use of cisplatin is compromised by severe nephrotoxicity and ototoxicity [202]. Cells in culture,

when exposed to cisplatin, as a part of this study, showed severe growth inhibition, where as apocynin did not show any inhibitory effects indicating a lack of inherent cytotoxic influence, towards both the cell lines. Also, apocynin did not show synergistic effects with cisplatin.

Prior exposure of both the cell lines to apocynin, in our treatments, could reduce the cell inhibition caused by cisplatin. Several studies have shown that the administration of antioxidants can reduce the side effects associated with cisplatin [99, 107, 129, 202]. Cisplatin treatment is associated with increased NADPH oxidase activity [203]. Apocynin, being a strong inhibitor of NADPH oxidase could have lowered the cisplatin cytotoxicity observed in the current study. NADPH oxidase inhibitors were earlier found to be significant in protecting cells from cisplatin-induced toxicity mediated by a significant reduction in ROS generation [202, 204]. Another mechanism by which cisplatin induces nephrotoxicity is depletion of the intracellular GSH [205]. Supplementing with cysteine, a glutathione precursor, also reduced the cisplatin-induced genotoxicity [86]. Apocynin is also proposed to be an enhancer of intracellular GSH [206], which was also observed in the hepatic tissues of experimental animals in the current study.

Agents which can offer selective protection to normal cells without interfering with the effectiveness of therapeutic drugs on cancer cells are best suited inclusion as adjuvants chemopreventive and chemoprotective strategies [101, 207, 208]. Natural substances of varied nature were shown to possess selective anticytotoxic efficiency towards normal cells rather than cancer cells, using the MTT assay. The methanolic extract of *Solanum nigrum* showed superior activity on *HeLa* cell line relatively low toxicity to *Vero* cells [100]. Studies by Senthilraja and Kathiresan [2015] prove marine yeast to have apoptotic effects on cancer cells and little activity on normal cells [101]. Emodin has synergistic

effects on the cytotoxicity of four different drugs in prostate cancer cell lines, but poses no threat to normal fibroblasts [207]. Mangiferin in combination with low non cytotoxic concentrations of cisplatin and 5-fluorouracil increased the cytotoxicity of these chemotherapeutic agents in mouse colon carcinoma cell lines without significant increase of cell death in CHO-K1cells [208]. In case of apocynin, we did not observe any selective cytoprotective activity, between the two studied cell lines. It showed similar effects towards both the cancer and normal cell lines. It is necessary to further elucidate this aspect by further studies, employing different cell lines and experimental conditions. Cytotoxic actions of antitumour drugs are in general facilitated by prominent increase of ROS generation; cells bearing a lower ROS status usually respond less to therapy. Fine and careful modulation of cellular redox status is a prospective strategy to enhance sensitivity of cancer cells to drugs. The mechanisms underlying the modulation of the level of ROS have to be carefully designed to ascertain the right redox-modulating agents which are efficient and at the same time, cancer cell-selective [207]. To evaluate haemocompatibility of apocynin haemolysis assay was performed as a component of this research work. Biomaterials are capable of structural destabilization of phospholipid bilayer of the membrane and ensuing lysis of RBCs or by damaging the cytoskeletal components. The cytoskeleton of RBC comprises of many proteins like spectrin and actin. The breakdown of erythrocytes impairs the ability of this fluid connective tissue to carry oxygen to various tissues. Free radicals are capable of lysing erythrocytes by oxidative injury to the membranes and certain antioxidants are potent inhibitors of such damage [209]. Cisplatin induces negligible haemolysis, confirming that chromatin is its biological target [110]. The same was also observed in the haemolysis study carried out currently. Treatment with apocynin or a combination of apocynin and cisplatin also had minimal effect on haemolysis. Apocynin and its analogs like ethyl vanillin could inhibit oxidative haemolysis as reported in an earlier study [138]. Apocynin

does not show any aggravation of haemolytic properties of cisplatin. Our results point to the haemocompatibity of apocynin, and may be safely integrated into chemotherapy regimen.

We also evaluated DNA protecting strength of apocynin by *in vitro* DNA fragmentation assay. Substances with strong antioxidant nature are known to be capable of protecting DNA from ROS induced damage [105]. This, in turn, is a hindrance for carcinogenic and genotoxic substances [2]. Many natural substances have been shown act as potent antioxidants that prevented genotoxicity in bone marrow cells as well as DNA fragmentation [107, 210]. The current DNA fragmentation study indicated that apocynin offered protection against cisplatin imposed threat to DNA in cultured CHO-K1 cells, indicated by the absence of a smear in the DNA extracted from the cells treated with a combination of apocynin and cisplatin, where as cisplatin alone caused smearing. In an earlier report, apocynin had caused a profound drop in caspase-3 activity and fragmentation of nuclear DNA in cisplatin-treated group [79]. This was attributed to the capability of apocynin to ease the severity of the intracellular stress favouring apoptotic activation. Apocynin was also found to be efficient in protecting DNA of oesophageal cells from damage, by inhibition of ROS generation and increasing the DNA repair capacity [211, 212]. The ability of apocynin in protecting the cultured CHO-K1 cell DNA may be due to its ability to inhibit cisplatin evoked NADPH oxidase activity and the concomitant ROS production [203].

The results of this study indicate the antigenotoxic and anticytotoxic efficiency of apocynin under the studied experimental conditions. Apocynin *per se* did not demonstrate any genotoxicity towards any of the investigated endpoints. These findings also warrant further studies to get deeper insight into the prospects of apocynin as an antigenotoxic compound. Apocynin could be a

potential antigenotoxic, anticytotoxic and chemopreventive agent against the effect of different environmental, industrial and chemotherapeutic genotoxic substances. It also shows prospective application as an adjuvant in chemotherapeutic interventions.

5.2 Genotoxic and Antigenotoxic Influences of Diosgenin

Diosgenin is evident in its applications against various metabolic disorders including dyslipidemia, diabetes and liver disorders, different types of tumours occurring in colon, breast, cervix, prostate, liver and other organs and other human ailments like dementia [32, 34, 173]. Diosgenin and other saponins have been shown to target multiple stages of cancer development, including tumour growth, angiogenesis, invasion, and immunosuppression in various *in vivo and in vitro* tumour cells [168, 170, 177, 213, 214]. In view of the immense therapeutic potential displayed by diosgenin, it is evidently necessary to understand the genotoxic and antigenotoxic nature of this compound. The present study aimed to reveal the nature of diosgenin towards induction of genotoxic damage using the Mouse erythrocyte micronuclei formation assay. CP was employed to induce genotoxic conditions in the experimental system. The other indicators of genotoxic damage that were studied include the level of hepatic lipid peroxidation, level of hepatic GSH and the haematological parameters.

In the current study against cyclophosphamide induced genotoxicity in mice bone marrow, diosgenin showed significant antigenotoxic activity, marked by reduction in the frequency of MnPCE caused by exposure to CP, in the experimental animals. Earlier Studies revealed that diosgenin has potent anticlastogenic effects on 7,12- dimethylbenz(a)anthracene treated hamsters[175]. Similar reports are provided by anticlastogenic effect of aqueous extract from water yam *Dioscorea alata* [215]. Diosgenin *per se,* did not aggravate formation of Mn.

Different modes of action are proposed for the biological effects displayed by diosgenin, involving a wide range of cellular modulating mechanisms [32]. It induces cell cycle arrest and apoptosis in osteosarcoma cells [33], diminishes matrix metalloproteinases and inhibits metastasis of prostate cancer cells [216] and suppresses 3-hydroxy-3-methylglutaryl CoA reductase expression in human colon cancer cells [217]. The antigenotoxicity of diosgenin, visible in our study, may probably be due to its antioxidant ability and also its effect on enhancing the GSH levels [170] which are also observed in the current study. As evidenced by other reports also, saponins with strong antioxidant capacities are capable of countering genotoxic damage caused by chemotherapeutic agents [170, 214, 215]. The modulatory effects of diosgenin against CP genotoxicity need further exploration to understand the interlaying molecular mechanisms.

Diosgenin was potent in normalizing the loss of WBC imposed by CP. The results of an earlier study suggest that diosgenin can modulate lymphopoesis [173]. Other studies report that diosgenin and its analogues play a role in modulating inflammatory mediators [218] through mechanisms that are yet to be deciphered. It conferred resistance to lymphocyte DNA damage under oxidative stress [31]. The present experimental results suggest diosgenin can moderate the leucopenia induced by treating the experimental animals with CP. The observation that diosgenin *per se* did not cause a lowering of total WBC count suggests it to be safe on the hematopoetic system. Diosgenin also did not affect the total RBC numbers and HB percentage.

In the experiments performed to further analyse the antigenotoxic nature of this sapogenin, diosgenin pretreatment was seen to significantly trim down the levels of LPO in the liver, as observed by reduced MDA quantities. Diosgenin was shown to lower peroxidation induced by CP in the urinary bladder of

experimental animals in some previous reports also [169, 171, 175] which support our current observations.

An imbalance in the status of GSH in the liver homogenate is indicative of interruption of detoxification process by oxidative stress. The antiperoxidative potential of reduced glutathione on cyclophosphamide-induced lipid peroxidation was clearly demonstrated by Ray *et al*., (2010) [199]. In our experiments, diosgenin extended a protective effect on non-enzymatic antioxidant GSH besides the level of LPO. The GSH level was lowered significantly by CP treatment. The point that diosgenin is competent in offsetting CP-induced oxidative stress, is supported by the increased GSH and the decreased LPO levels seen in our study. These results are in agreement with other previous reports [171]. The molecular mechanism underlying this protective ability could be directly related to its notable potential to scavenge radical species [169]. Similar effects of diosgenin are reported by other workers where it reduced the plasma level of total cholesterol and conferred resistance to lymphocyte DNA damage under oxidative stress [31].

In the cell growth inhibition assays performed currently to evaluate cytotoxic and anticytotoxic nature of diosgenin, it has shown a significant cytotoxic action towards the cancer cell line HepG2. It also showed a synergistic effect with cisplatin by showing a dose dependent increase in the cisplatin induced cytotoxic activity on HepG2 cells, higher concentration of 100μM being the most effective, of the three tested concentrations. Plant extracts containing diosgenin and other steroid saponin constituents showed *in vivo* and *in vitro* anticancer activity [214]. Studies have shown that diosgenin possesses potential prooxidant properties causing growth inhibition of Hep2 cells[103] and HepG2 cells[168], mediated by increased ROS generation and lipid peroxidation.

Diosgenin is shown to affect manifold steps of cell signalling associated with cell growth, differentiation and programmed cell death [32] in its chemopreventive activity. It was shown to suppress the expression of enzymes like metalloproteinases and kinases and also signalling molecules like nuclear factor kappa B (NF-κB) [216]. Diosgenin had enhanced the apoptotic effects of anticancer drugs paclitaxel and doxorubicin by lowering the production of STAT3-regulated gene products [172]. Different molecular mechanisms of action, responsible for the antiproliferative action of these steroidal saponins *in vitro*, on other cell lines are reported by other researchers also [32, 170, 176, 217].

On the other hand, reports of anticlastogenic nature of diosgenin and plant extracts containing steroidal saponins, mediated by antioxidant nature and enhancement of cellular antioxidants in different biological model systems are provided by other workers [175,215, 219]. Studies by Das & Bharali (2014) reveal that diosgenin possesses significant free radical scavenging ability and acts as a bifunctional inducer of xenobiotic detoxifying enzymes [169]. In this context, a review of a number of scientific reports shows both anticlastogenic nature of diosgenin, especially *in vivo* and its cytotoxic nature on cancer cells *in vitro*. In the current study also similar results were obtained. Diosgenin is a natural compound with immense potential for therapeutic applications across a wide range of metabolic disorders and cancers [34]. The molecular mechanism of antigenotoxic activity as well as the synergistic cytotoxic ability of diosgenin with cisplatin can be further explored to understand its chemotherapeutic potential. This could prove valuable in clinical use of diosgenin against cancer and other metabolic disorders [173].

The other cell line employed in our present research was CHO-K1 whose growth was not affected by diosgenin, in any manner. It did not inhibit the growth of these cells in culture nor had

synergistic effects with cisplatin; at the same time it did not affect the cytotoxic action of cisplatin on these cells, indicating a lack of cytoprotective effect. There are no previous reports of influence of diosgenin on non cancerous cell cultures. We observed that though this sapogenin is able to cause death of HepG2 cells, it did not cause growth inhibition in the normal cells. This is an important observation in enabling the chemotherapeutic usage of diosgenin. In view of the previous reports, our findings demonstrate that diosgenin is bestowed with promising attributes that need further exploration and make it a potential candidate for application as antigenotoxic as well as chemotherapeutic agent in the chemotherapy strategies.

❑❑❑

CHAPTER - 6

SUMMARY AND CONCLUSIONS

6.1 Summary

Genotoxic agents are substances capable of causing direct damage to genetic material, leading to mutation and clastogenic disturbances, which further can lead to occurrence of degenerative diseases and cancer. Reduction in the incidence of such damage can lead to reduction in the occurrence of mutation based diseases. Exposure to environmental pollutants, industrial chemicals and effluents, heavy metals, cytotoxic drugs and other genotoxicants might result in the development of different kinds of cancer. Toxicity of substances is manifested in diverse forms like DNA damage, formation of micronuclei, Chromosomal aberrations, cell death or abnormal cell growth leading to tumour formation. Contact with these agents is bound to happen time and again and creates a threat to human health. Cancer chemotherapy is one of such situations where toxicity to non cancerous tissues is a major problematic issue. The need to overcome the genotoxic side effects of chemotherapeutic as well as other drugs is impending. Use of natural

compounds with antioxidative, antigenotoxic, antimutagenic and cytoprotective abilities has gained importance globally. Understanding the inherent genotoxic/antigenotoxic as well as cytotoxic/cytoprotective of these natural substances is of utmost importance, to employ these substances for their chemotherapeutic and chemopreventive applications. With this background, we attempted to evaluate the genotoxic and antigenotoxic properties and also the cytotoxic/ cytoprotective abilities of two plant active constituents namely apocynin and diosgenin, in this thesis. Apocynin is a strong inhibitor of the enzyme NADPH oxidase and is bestowed with several pharmacological applications. Diosgenin also, has well known therapeutic applications. Reports on the antigenotoxic properties and cytoprotective abilities of these two compounds are limited. Hence, we studied these two compounds.

The incidence of micronuclei in the polychromatic erythrocytes of mice bone marrow was studied, as an indicator of genotoxic damage inflicted on the DNA by external agents. The chemotherapy agent cyclophosphamide was employed to induce genotoxic conditions *in vivo*. Two biomarkers of oxidative stress, namely cellular status of lipid peroxidation and reduced glutathione in the hepatic tissues were analysed since oxidative stress is a major mechanism of genotoxicity. Higher levels of oxidative stress are conducive for genotoxic damage. The influence of the test compounds on the total count of WBC, RBC and haemoglobin content was also studied. The efficiency of the test compounds in terms of cytotoxicity and cytoprotective/anticytotoxic nature was established by performing MTT assay with two different types of cell lines, CHO-K1 and HepG2, to ascertain their safety, against the cytotoxicity induced by chemotherapy drug cisplatin. The protective abilities of the plant compound apocynin against oxidative DNA damage was also studied by agarose gel electrophoresis. Apocynin was also tested for any inherent haemolytic properties. The effect of the selected plant compound apocynin on occurrence

of chromosomal aberrations in the root tip meristems of *Allium cepa*, as an indicator of genotoxic and antigenotoxic properties, in the presence of suitable negative and positive controls, was also studied.

In the various experimental studies we carried out, Apocynin showed –

- Significant antigenotoxic activity in the mice bone marrow micronucleus assay by reducing Mn frequency induced by cyclophosphamide.
- Efficient restoration of total WBC count against CP induced luecopenic conditions in mice.
- Protection against lipid peroxidative damage and increase in cellular antioxidant GSH in the liver homogenates.
- Significant reduction in the chromosomal aberrations induced by CP in *Allium* root meristems.
- Exhibited no inherent cytotoxic activity towards both HepG2 and CHO-K1.
- Counteracted the cytotoxicity of cisplatin in cell lines HepG2 and CHO-K1. It did not show any selectivity towards the normal cells in its cytoprotective action.
- Protection against oxidative DNA damage caused by cisplatin *in vitro*.
- Showed no haemolytic activity, indicating it to be safe on the blood system.

Our study also revealed that diosgenin shows –

- Significant antigenotoxic activity in the mice bone marrow micronucleus assay by reducing Mn frequency induced by cyclophosphamide.
- Efficient restoration of total WBC count against CP induced luecopenic conditions in mice.
- Protection against lipid peroxidative damage and increase in GSH in the liver homogenates.

- Significant cytotoxicity towards HepG2 cells in culture but no cytotoxicity towards CHO-K1 cells.
- Significant enhancement of cytotoxic action of cisplatin on cancer cell line HepG2 but does not affect the cytotoxicity of cisplatin on CHO-K1. It does not exert any cytoprotective effect towards both the cell lines.

6.2 Conclusions

- The results of this study indicate the potential antigenotoxic and anticytotoxic efficiency of apocynin under the studied experimental conditions.
- With careful further studies, apocynin has the potential to be employed as antigenotoxic/ chemopreventive agent in chemotherapy strategies, to prevent clastogenic damages induced by chemotherapeutic drugs. It also does not pose any cytotoxic or genotoxic risk.
- Our findings point that diosgenin is bestowed with promising qualities as an antigenotoxic agent that need further exploration and make it a potential candidate for application as an adjuvant in the chemotherapy strategies to manage the clastogenicity of drugs.
- Our observation that diosgenin has cytotoxicity only towards cancer cells and not towards normal cells makes it a potent candidate for development into a therapeutic agent.

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